Isolation and Characterisation of New R-protein Variants Encoded at the Barley *Mla* Locus that Specify Resistance Against the Fungus Powdery Mildew

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Summary

The *Mla* locus in barley (*Hordeum vulgare* ssp. *vulgare*) contains a large number of alleles with distinct recognition specificities against isolates of the fungal pathogen powdery mildew (*Blumeria graminis* f. sp. *hordei*). Classical genetic studies have found more than 30 *Mla* specificities in different barley cultivars. Previously, only six *Mla* alleles have been isolated and characterised at the molecular level. We isolated and characterised 23 new *Mla* sequences designated as candidate MLA cDNAs using a PCR-based approach. Thus, *Mla* currently contains a total of 29 different variants. Functional activity was demonstrated for 13 of the 23 candidate MLA cDNAs. Four *Mla* sequences encoded N-terminal coiled-coil (CC) regions that differed significantly from the N-terminus conserved among the other 25 MLA proteins. The MLA proteins are highly polymorphic in the C-terminal LRR (leucine-rich repeat) domain. Investigation of this region revealed 30 positively selected sites that lie mostly in the variable x positions of the 15 LxxLxLxx motifs. As the LRR domain is possibly determining recognition specificity, the large diversity of MLA will help to precisely identify the sequence requirements for the detection of the pathogenic effector proteins.

Zusammenfassung

Der *Mla*-Locus von Gerste (*Hordeum vulgare* ssp. *vulgare*) besitzt eine grosse Anzahl an Allelen mit unterschiedlichen Spezifitäten gegen Pilzisolate von Gerstenmehltau (*Blumeria graminis* f. sp. *hordei*). Mit Hilfe von klassischen genetischen Studien wurden über 30 unterschiedliche *Mla*-Allele in verschiedenen Gerstensorten entdeckt. Bis anhin waren nur sechs *Mla*-Allele isoliert und auf molekularer Stufe charakterisiert worden. Wir haben 23 neue *Mla*-Sequenzen, die wir als MLA-cDNA-Kandidaten bezeichnen, mit einem PCR-basierten Ansatz isoliert und charakterisiert. Momentan besitzt *Mla* somit 29 unterschiedliche Varianten. Funktionelle Aktivität konnte für 13 der 23 MLA-cDNAs gezeigt werden. Vier *Mla*-Sequenzen kodierten N-terminale Coiled-coil-(CC-) Domänen, welche sich deutlich vom konservierten N-Terminus der restlichen 25 MLA-Proteine unterscheiden. Die MLA-Proteine sind in der C-terminalen LRR-Domäne (Leucine-rich repeat) hoch polymorph. Eine Untersuchung dieser Region ergab 30 positiv selektionierte Positionen, welche vorwiegend in den variablen x-Positionen der 15 LxxLxLxx-Motive liegen. Da die LRR-Domäne möglicherweise die Erkennunsspezifität bestimmt, wird die grosse Anzahl an isolierten *Mla*-Sequenzen helfen, die Sequenzanforderungen für die Erkennung der Effektorproteine des Pathogens genau zu identifizieren.

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1. Introduction

1.1 Plant defence mechanisms

There are hundreds of plant pathogens including bacteria and fungi that infect plants, undergo proliferation and thus, cause sometimes severe plant diseases. Nevertheless, most plants are resistant against most pathogenic organisms due to their immune system which consists of several different defence strategies.

1.1.1. MAMP receptors

Plants have a basal defence system similar to the innate immune system in mammals, consisting of membrane-associated pattern recognition receptors (PRRs) that detect evolutionarily conserved molecules produced by pathogens called MAMPs (microbe-associated molecular patterns; SHEN and SCHULZE-LEFERT 2007). A second, adaptive defence system specific for plants consists of resistance (*R*) genes (Figure 1). The majority of these *R* genes encode intracellular proteins of the NB-LRR (nucleotide binding and leucine-rich repeat) type (SHEN *et al.* 2007). They are classified according to their N-terminal domain in TIR-NB-LRR (Toll-like) and CC-NB-LRR (coiled-coil) type of proteins (DANGL and JONES 2001). These R proteins can detect pathogen-derived effector molecules either directly or indirectly and hence trigger a hypersensitive response (HR), a fast cell death of the infected host cell, through a mostly unknown mechanism (JONES and DANGL 2006). HR is discussed in more detail in chapter 1.1.3.

In contrast to the *R* gene-dependent defence, the response to MAMPs does not trigger an HR. General elicitors (MAMPs) include lipopolysaccharides or flagellin from bacteria or chitin from fungi. In *Arabidopsis*, a 22 residues long peptide, flg22 is recognized by the receptor FLS2 (flagellin-sensing 2; Figure 1; ZIPFEL *et al.* 2004). A second receptor, EFR, recognizes two peptides (elf18 and elf26) of the bacterial elongation factor EF-Tu (ZIPFEL *et al.* 2006). Both, FLS2 and EFR belong to the receptor-like kinases (RLKs) that are composed of an extracellular ligand-binding domain, a single transmembrane domain and an intracellular serine/threonine kinase domain (ALTENBACH and ROBATZEK 2007). Recently, it was demonstrated that FLS2 and EFR need a co-receptor called BAK1 (BRI1-associated receptor kinase 1), also an LRR receptor-like kinase (CHINCHILLA *et al.* 2007). BAK1 seems to be essential for the regulation of various receptors, including the plant hormone receptor BRI1 (CHINCHILLA *et al.* 2007).

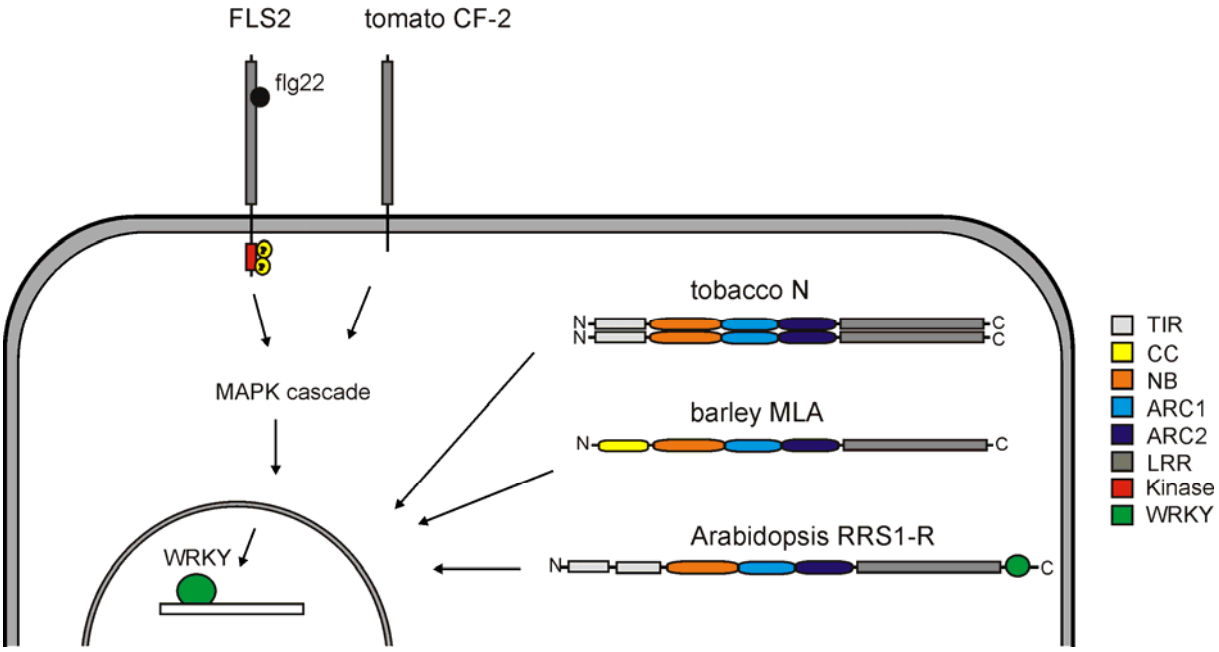


Figure 1: Illustration of typical members of the three R protein classes and the receptor-like kinases. All protein domains and their related colours are indicated on the right side of the figure. FLS2 is a member of the receptor-like kinases, which is activated upon MAMP recognition of the flagellin peptide flg22. The R protein tomato Cf-2 belongs to membrane-bound receptor-like proteins. Tobacco N is a member of the TIR-NB-LRR type, which was demonstrated to oligomerize upon activation (MESTRE and BAULCOMBE 2006). Barley MLA belongs to the CC-NB-LRR class and Arabidopsis RRS1-R carries a C-terminal WRKY domain. All three R proteins enter the nucleus upon activation (BURCH-SMITH *et al.* 2007; DESLANDES *et al.* 2003; SHEN *et al.* 2007). The MAPK pathway and WRKY transcription factors (green) are involved in immune responses.

In humans, the Toll-like receptors (TLRs) recognize MAMPs like flagellin or chitin. These receptors oligomerize upon ligand binding, as demonstrated for TLR3 (BELL *et al.* 2006). Thus, it is likely that plant receptor like kinases also undergo receptor oligomerization to stabilize ligand binding or to trigger signalling cascades (ALTENBACH and ROBATZEK 2007). A plant MAP kinase cascade and the transcription factors WRKY22 and WRKY29 belong to the downstream factors induced by the binding of flg22 to FLS2 (ASAI *et al.* 2002). It was demonstrated that FLS2-GFP fusion constructs localize to the plasma membrane and to intracellular vesicles. The authors suggested that FLS2 is degraded via endocytotic pathways after ligand binding (ROBATZEK *et al.* 2006).

1.1.2. Non-host resistance in plants

Most pathogens are host-specific and thus, can not infect more than one plant species. E.g. *Arabidopsis thaliana* is not infected by barley powdery mildew (*B. graminis f. sp. hordei*). These so-called non-host defence mechanisms include strategies such as the thickening of the cell wall or the production of antimicrobial metabolites (JONES and DANGL 2006). A screen of

Arabidopsis mutants that show increased penetration of barley powdery mildew spores identified three mutations in genes, called *pen1*, *pen2* and *pen3* (COLLINS *et al.* 2003). PEN2 is a β-glycosyl hydrolase which localizes to peroxisomes at fungal entry sites (LIPKA *et al.* 2005). It was postulated that PEN2 accumulates near the infection site and converts an unknown substrate to a toxic product (STEIN *et al.* 2006). PEN3 encodes an ABC transporter of the plasma membrane, which accumulates at the fungal entry site. It was suggested that PEN3 exports defence compounds that are produced by PEN2 (STEIN *et al.* 2006).

During exocytosis, vesicles are transported to, and fused with, the target membrane. This process is thought to be induced by complex formation between (t)-SNAREs (like syntaxins and SNAP25 proteins) and (v)-SNARES (the VAMP family; KWON *et al.* 2008). PEN1 is a syntaxin that forms a ternary complex with the SNAP25 homologue SNAP33 and with VAMP721/722 to mediate vesicle fusion (KWON *et al.* 2008; PAJONK *et al.* 2008). The accumulation of sterols induced by the penetration of powdery mildew spores has also been demonstrated (BHAT *et al.* 2005). Thus, like PEN2 and PEN3, PEN1 is assumed to accumulate at the pathogen entry sites in membrane-specific structures termed sterol-enriched lipid rafts (KWON *et al.* 2008). PEN2 and PEN3 act in the same pathway, whereas PEN1 is involved in a different cascade (KWON *et al.* 2008).

1.1.3. The mechanisms of hypersensitive response

Several features characteristic for basal immunity are shared with pathways involved in NB-LRR gene-mediated adaptive responses: the transcriptional activation of pathogen-related (PR) genes, the biosynthesis of anti-microbial toxins or MAPK signalling (CHUNG *et al*., 2008). One of them, the production of reactive oxygen intermediates (ROI), is specifically important for NB-LRR-induced resistance, called the oxidative burst (GLAZEBROOK 2005). The mechanisms inducing a programmed cell death (termed hypersensitive response, HR) are characteristic for NB-LRR genes. HR is part of the adaptive response and is not triggered by the basal defence machinery.

HR is a highly efficient defence response specifically against biotrophic pathogens, which depend on living host tissue. Both the pathogen and the infected host cell die during this process (GLAZEBROOK 2005). In contrast, necrotrophic pathogens live on dead host tissue and are thus thought to profit from host cell death. It is assumed that defence against necrotrophs

depends on jasmonic acid and ethylene signalling, whereas salicylic acid pathways are involved in biotrophic resistance responses, due to the association with the HR. It was shown that there is a crosstalk between salicylic acid and jasmonic acid pathways. Apart from strict biotrophs and necrotrophs, several pathogens can switch between both lifestyles, depending on the stage of their life cycles or environmental conditions, and are thus termed hemi-biotrophs (GLAZEBROOK 2005).

1.2 CC/TIR-NB-LRR resistance proteins

1.2.1. The CC and TIR domains

The N-terminus of CC-NB-LRR proteins is predicted to contain a coiled-coil (CC) motif, which describes α-helices with repetitive hydrophobic residues that form a hydrophobic core in the protein dimer. For example, the R protein MLA interacts through its predicted coiled-coil with the transcription factors HvWRKY1 and HvWRKY2 (SHEN *et al.* 2007). Two R proteins interact through their CC domains with so-called guardee proteins, which are suggested to be the target of pathogenic effectors: RPM1 with RIN4 and RPS5 with PBS1 in *A. thaliana* (ADE *et al.* 2007; MACKEY *et al.* 2002). The CC domain of two other R proteins binds to proteins that may be involved in downstream signalling, namely tomato Prf with the kinase Pto and potato Rx with the GTPase-activating protein RanGAP2 (MUCYN *et al.* 2006; SACCO *et al.* 2007). A DNA-binding motif known as the BED-finger, a zinc finger motif, was found in the CC domains of several R proteins (TAMELING and TAKKEN 2008). This domain was discovered in transcriptional regulators and chromatin insulators of *Drosophila,* and thus, it is likely that it plays a similar role for R proteins (TAMELING and TAKKEN 2008). Besides its involvement in effector recognition and signalling, the CC domain seems to interact with the NB-LRR domains. Recently, a highly conserved EDVID motif was discovered that appears to be important for intramolecular folding of CC-NB-LRR proteins (RAIRDAN *et al.* 2008).

The Toll-interleukin receptor (TIR) domain obtained its name due from homology to the intracellular signalling domain of the Toll and interleukine receptors (DANGL and JONES 2001). Oligomerization of the TIR domain was shown for the tobacco N resistance protein (MESTRE and BAULCOMBE 2006). Thus, it is possible that oligomerization in response to pathogen elicitors is a general feature of TIR-NB-LRR proteins. It seems that the TIR domain is involved in recognition, as the CC domain in CC-NB-LRR proteins. It was demonstrated

that the tobacco N protein interacts with its cognate effector protein p50 through the TIR domain (BURCH-SMITH *et al.* 2007).

1.2.2. The NB-ARC domain

The nucleotide-binding site (NB-ARC) domain consists of three different subdomains, the nucleotide-binding domain (NBS), the helical domain (ARC1) and the extended winged helix domain (ARC2) (RAIRDAN and MOFFETT 2007; RAIRDAN *et al.* 2008). The NB-ARC domain is shared between plant R proteins and human Apaf-1, the apoptotic protease-activating factor 1, and CED4, the Apaf-1 homologue in *Caenorhabditis elegans* (TAKKEN *et al.* 2006). NB-LRR proteins, Apaf-1, CED-4 and the mammalian NACHT-LRR proteins (NLRs) like CIITA, belong to the family of STAND ATPases (RAIRDAN and MOFFETT 2007). Recently, the crystal structures of ADP-bound Apaf-1 and ATP-bound CED-4 were solved (RIEDL *et al.* 2005; YAN *et al.* 2004).

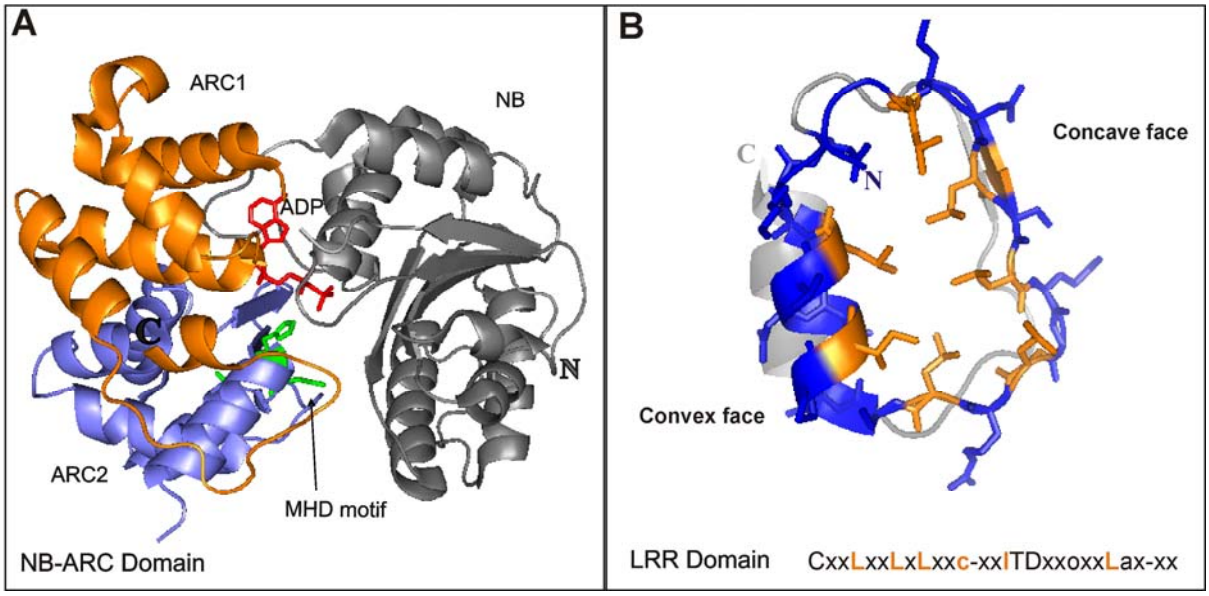


Figure 2: Representative structures of NB-ARC and LRR domains. A) Crystal structure of human Apaf-1 with bound ADP (pdb entry 1z6t; residues 1-450; RIEDL *et al.* 2005). Red: ADP. Gray: NBS domain (108-284). Orange: ARC1 domain (285-365). Blue: ARC2 domain (366-450) with the highly conserved MHD motif (green). B) The 4th and 5th LRR of the crystal structure from Skp2 (pdb entry 1fqv; residues 118 – 266; SCHULMAN *et al.* 2000). Skp2 belongs to the same class of LRR proteins as plant R proteins. Orange: hydrophobic residues (mostly leucine) forming a hydrophobic core. Blue: the variable x position of the LxxLxLxx motif. The conserved LxxLxLxx motif of the LRRs is situated on the concave solvent-exposed site consisting of a short beta sheet. An alpha helix lies on the convex inner face. The picture was generated with the programs PyMOL and CorelDraw.

The NB-ARC domain is proposed to be a molecular switch induced by ATP/ADP exchange and the hydrolysis of a ATP (VAN OOIJEN *et al.* 2007). The Walker A (also known as the Kinase 1 motif) and the Walker B (Kinase 2) motifs, which form the NTP-binding pocket, are

the most conserved patterns (TAKKEN *et al.* 2006). Mutation of the Walker A or Walker B motif resulted in inactivation of tobacco Rx and *Arabidopsis* RPS2, indicating its importance in ATP binding (BENDAHMANE *et al.* 2002; TAO *et al.* 2000). It was demonstrated that the two tomato R proteins I-2 and Mi-1 bind ATP *in vitro*, whereas proteins with a mutated Walker A motif did not bind ATP anymore (TAMELING *et al.* 2002; TAMELING *et al.* 2006). It is proposed that the ATP-binding form is the active state of NB-LRR proteins because autoactive mutants of I-2, which spontaneously induced HR, were only involved in ATP hydrolysis but not in ATP binding (TAMELING *et al.* 2006). A different motif in R proteins, RNBS-B, is thought to be involved in γ-phosphate binding (TAKKEN *et al.* 2006). The function of the RNBS-C, LxxLL and RNBS-A motifs, which are characteristic for plants, are unknown (RAIRDAN and MOFFETT 2007).

The ARC1 domain mediates intramolecular folding by interaction with the LRR domain, as demonstrated for potato Rx (RAIRDAN and MOFFETT 2006). The LRR domain of Rx interacts with its own CC-NB-ARC domain (MOFFETT *et al.* 2002). The ARC1 subdomain contains a GLPL motif, which was shown to be within the NTP-binding pocket in case of Apaf-1 (Figure 2A).

The ARC2 domain containing the highly conserved MHD motif seems to be involved in the regulation of ATP binding and hydrolysis, which is assumed to activate the NB-LRR proteins (RAIRDAN and MOFFETT 2007). For Apaf-1, the histidine residue of the MHD motif was shown to contribute a hydrogen bond to the β-phosphate of the bound ADP (Figure 2A; RIEDL *et al.* 2005). For several R proteins like tomato I-2 and Mi-1, potato Rx or flax L6, autoactive mutants were generated by replacing the aspartate or histidine residues (RAIRDAN and MOFFETT 2007).

1.2.3. The LRR domain

Leucine-rich repeat (LRR) domains encoded by resistance genes are thought to determine recognition specificity (DEYOUNG and INNES 2006). This assumption is based on studies about the involvement of LRR domains of several protein families like the mammalian Toll-like receptors, which are involved in ligand binding or protein-protein interactions (BELLA *et al.* 2008). A typical leucine-rich repeat consists of 20 to 29 residues that form a β strand followed by an α helix, which are arranged parallel to a common axis (Figure 2B; KAJAVA *et al.* 1995). The conserved segment of 11 amino acids (LxxLxLxxN/CxL, where x stands for any residue and the L position can be occupied by any hydrophobic amino acid) of the β-

strand/β-turn motif lies on the concave face (KOBE and KAJAVA 2001). The leucine or related residues of the LRR motif point inward and build a hydrophobic core that gives a lateral stabilization to the architecture, as deduced from the crystal structure of the human Toll-like receptor TLR3 (CHOE *et al.* 2005). The convex face of the LRR is formed by an α-helix. The first crystallographically solved plant-specific LRR domain was obtained from the plant cell wall protein PGIP (polygalacturonase-inhibiting protein) (DI MATTEO *et al.* 2003). As no crystal structure has been solved for any NB-LRR type resistance protein, the LRR domain of Skp2 is often used as a model for this subclass of LRR proteins (Figure 2B; KOBE and KAJAVA 2001).

Several studies revealed that residues of the LxxLxLxx β-sheet motif in the LRR domain are under stronger positive selection than other parts of the proteins (Table 1; BOTELLA *et al.* 1998; DODDS *et al.* 2001a; DUNNING *et al.* 2007; ELLIS *et al.* 1999; HUANG *et al.* 2008; LECKIE *et al.* 1999; MCDOWELL *et al.* 1998; MEYERS *et al.* 1998; MONDRAGON-PALOMINO *et al.* 2002; NOEL *et al.* 1999; ROSE *et al.* 2004; YAHIAOUI *et al.* 2006). In particular, diversifying selection was shown to occur in NB-LRR genes with allelic series like *Arabidopsis RPP13*, the flax *L* gene, or wheat *Pm3* as nonsynonymous substitutions are overrepresented in comparison to changes in synonymous sites (ELLIS *et al.* 1999; ROSE *et al.* 2004; YAHIAOUI *et al.* 2006).

1.2.4. The guard and decoy model

Several NB-LRR proteins recognize effectors indirectly by monitoring their host target protein, called the guardee, and thereby obtaining information about the effector's activity such as binding or target modification (JONES and DANGL 2006). This indirect recognition is known as the guard hypothesis, first postulated to explain the resistance mechanisms involved in AvrPto recognition by the Pto-Prf system (VAN DER BIEZEN and JONES 1998). The theory was then further generalized so that it can be applied to several NB-LRR proteins (DANGL and JONES 2001). Its main characteristics are the following: The interaction between an effector and its host target is monitored by an R protein, which gets activated to initiate a resistance response. If the host protein is not guarded by an R protein, the pathogen will grow and the disease develops (DANGL and JONES 2001). This assumption raises the possibility that several effectors can manipulate the same host target. This could result in the evolution of different

NB-LRR proteins that monitor the same guardee but recognize different types of activity (JONES and DANGL 2006).

Three NB-LRR proteins that are proposed to recognize effectors indirectly were shown to be conserved and under balancing selection, which means that allelic polymorphism is maintained within a population (Table 1; BENT and MACKEY 2007; MCDOWELL and SIMON 2006; STAHL *et al.* 1999; TIAN *et al.* 2002). One of them, *Arabidopsis RPS5*, is a single-copy gene that shows no allelic variety and detects cleavage of the protein kinase PBS1 by the *P. syringae* effector AvrPphB, a cysteine protease (SHAO *et al.* 2003; TIAN *et al.* 2002). A second example of an R protein that is under balancing selection is RPM1. It is predicted to recognize phosphorylation of the guardee RIN4 by the two unrelated *Pseudomonas syringae* effectors AvrRpm1 and AvrB (MACKEY *et al.* 2002; STAHL *et al.* 1999). A third effector, AvrRpt2, is a protease that cleaves the guardee RIN4 and thereby activates the NB-LRR gene *RPS2*. Polymorphisms at the *RPS2* locus have been investigated, and the authors postulate that *RPS2* fits best to the model of balancing selection (MAURICIO *et al.* 2003). The guardee RIN4 is proposed to be a negative regulator of MAMP-induced signalling because the corresponding cascades are inhibited by the RIN4 overexpression and enhanced by RIN4 absence (KIM *et al.* 2005).

The tomato R protein Pto, a Ser/Thr kinase, was the first *R* gene to be cloned that gives a gene-for-gene resistance against a pathogen (PEDLEY and MARTIN 2003). Pto depends on a interaction partner, the CC-NB-LRR protein Prf (PEDLEY and MARTIN 2003). Recently, co-immunoprecipitation experiments showed a physical interaction between the kinase Pto and the N-terminal region of the R protein tomato Prf (MUCYN *et al.* 2006). It was concluded that the Pto-Prf complex regulates immune signalling as a molecular switch (MUCYN *et al.* 2006). Additionally, yeast two-hybrid assays demonstrated that the kinase Pto interacts directly with the two unrelated effectors AvrPto and AvrPtoB (KIM *et al.* 2002; SCOFIELD *et al.* 1996). Both, AvrPtoB and AvrPto inhibit HR-based cell death (ABRAMOVITCH *et al.* 2003; XIANG *et al.* 2008). The effector AvrPtoB functions as an E3 ubiquitin ligase and is therefore suggested to target host proteins for degradation (ABRAMOVITCH *et al.* 2003). Recently, the solved crystal structure of the Pto-AvrPto complex showed how the effector inhibits the Pto kinase *in vitro* (XING *et al.* 2007). The effector AvrPto seems to have other targets besides the kinase Pto, as it was recently demonstrated that it inhibits the autophosphorylation of FLS2 and EFR (XIANG *et al.* 2008). In a yeast three-hybrid screen using the effector AvrPto and the kinase Pto as baits, the protein kinase Adi3 was identified and subsequently shown to be

phosphorylated by Pto. Adi3 is proposed to be involved in the regulation of plant cell death (DEVARENNE *et al.* 2006).

The guard model of indirect recognition fits well with the Pto-Prf system, where two unrelated effectors AvrPto and AvrPtoB are recognized by the same R protein Prf. Guardee proteins are predicted to be conserved virulence targets, but this seems to contrast with the absence of the kinase Pto in susceptible cultivars, in which the effector AvrPto is still virulent (MUCYN *et al.* 2006; RAIRDAN and MOFFETT 2007). Thus, a new model was recently suggested for the Pto-Prf system, called the decoy model (RAIRDAN and MOFFETT 2007; VAN DER HOORN and KAMOUN 2008). The decoy is postulated to be a mimic of the effector target without any other important function in disease establishment. Thus, the resistance response is initiated upon monitoring of decoy modifications by the corresponding NB-LRR protein (VAN DER HOORN and KAMOUN 2008). In this theory, Pto is a mimic of the kinase domain of FLS2 instead of a virulence target, and it gets activated in presence of the effector AvrPto to initiate an HR (RAIRDAN and MOFFETT 2007).

A second interesting example for the decoy model are the pepper *R* gene *Bs3* and its cognate effector *AvrBs3* from *Xanthomonas campestris pv. vesicatoria* (VAN DER HOORN and KAMOUN 2008). AvrBs3 mimics a eukaryotic transcription factor by binding and activating the promoter of Upa20, a protein that regulates cell size (KAY *et al.* 2007). Resistant pepper plants contain the *R* gene *Bs3* encoding a flavin monooxygenase, which gets activated not only by *Upa20,* but also by the effector AvrBs3 (ROMER *et al.* 2007). Because *Bs3* is not expressed in the absence of AvrBs3, It seems that *Bs3* has no other obvious function than detecting AvrBs3 activity (ROMER *et al.* 2007). Thus, *Bs3* is proposed to be a decoy by mimicking the AvrBs3 target *Upa20* (VAN DER HOORN and KAMOUN 2008).

1.2.5. Direct recognition of the effector

Several studies revealed that residues of the solvent-exposed LxxLxLxx β-sheet motif in the LRR domain are under stronger positive selection than other parts of the R proteins (Table 1; DUNNING *et al.* 2007). In particular, diversifying selection (genetic variation is favoured) was shown for NB-LRR genes with allelic series like *Arabidopsis RPP13*, the flax *L* gene, or wheat *Pm3* as nonsynonymous substitutions are overrepresented in comparison to changes in synonymous sites (ELLIS *et al.* 1999; ROSE *et al.* 2004; YAHIAOUI *et al.* 2006). It was recently proposed that in cases of direct recognition, both the *R* gene and the corresponding gene of the

Table 1

The three classes of isolated NB-LRR resistance genes

Type	R protein	number of alleles	species	positive selection in the LxxLxLxx motif	effector	species	References
I	*Dm3*	9 genes, complex locus	Lettuce	yes	*Avr3*	*Bremia lactucae*	(MEYERS *et al.* 1998; SHEN *et al.* 2002)
I	*Pi-ta*	2 different haplotypes	Rice	yes	*AvrPi-ta*	*Magnaporthe grisea*	(HUANG *et al.* 2008; JIA *et al.* 2000)
I	*RPP13*	19, single gene	Arabidopsis	yes	*ATR13*	*Hyaloperonospora parasitica*	(ALLEN *et al.* 2004; ROSE *et al.* 2004)
I	*Mla*	28, complex locus	Barley	yes	AVR_{A10}	*Blumeria graminis*	Table 2, (RIDOUT *et al.* 2006)
I	*RPP8*	3, complex locus	Arabidopsis	yes	n.d.	*Hyaloperonospora parasitica*	(MCDOWELL *et al.* 1998)
I	*Pm3*	7	Wheat	yes	n.d.	*Blumeria graminis*	(YAHIAOUI *et al.* 2006)
I	*RPM1*	27	Arabidopsis	balancing selection	*AvrRpm1*, *AvrB*	*Pseudomonas syringae*	(MACKEY *et al.* 2002; STAHL *et al.* 1999)
I	*RPS5*	1 gene, no allelic variety	Arabidopsis	balancing selection	*AvrPphB*	*Pseudomonas syringae*	(SHAO *et al.* 2003; TIAN *et al.* 2002; WARREN *et al.* 1999)
I	*RPS2*	7, single gene locus	Arabidopsis	balancing selection	*AvrRpt2*	*Pseudomonas syringae*	(BENT *et al.* 1994; CAICEDO *et al.* 1999)
I	*Prf*	1 gene	Tomato	n.d.	*AvrPtoB*	*Pseudomonas syringae*	(JANJUSEVIC *et al.* 2006; SALMERON *et al.* 1996)

I	*Bs2*	1 gene	Tomato	n.d.	*AvrBs2*	*Xanthomonas campestris*	(SWORDS *et al.* 1996; TAI *et al.* 1999)
I	*R3a*	1 gene	Potato	n.d.	*Avr3a*	*Phytophthora infestans*	(ARMSTRONG *et al.* 2005; HUANG *et al.* 2005)
I	*Rx1*	1 gene	Potato	n.d.	coat particle	Potato Virus X	(BENDAHMANE *et al.* 1999)
I	*Rx2*	1 gene	Potato	n.d.	coat particle	Potato Virus X	(BENDAHMANE *et al.* 2000)
I	*HRT*	1 gene	Arabidopsis	n.d.	coat particle	Turnip crinkle virus, coat protein	(COOLEY *et al.* 2000)
I	*Gpa2*	1 gene, same locus as Rx	Potato	n.d.	n.d.	*Globodera pallida*	(VAN DER VOSSEN *et al.* 2000)
I	*R1*	1 gene	Potato	n.d.	n.d.	*Phytophthora infestans*	(BALLVORA *et al.* 2002)
I	*Pib*	1 gene, gene family	Rice	n.d.	n.d.	*Magnaporthe grisea*	(WANG *et al.* 1999)
I	*Cre3*	1 gene	Wheat	n.d.	n.d.	*Heterodera avenae*	(LAGUDAH *et al.* 1997)
I	*Rp1-D*	1 gene, complex gene locus	Maize	n.d.	n.d.	*Puccinia sorghi*	(COLLINS *et al.* 1999)
I	*Xa1*	1 gene, single-copy gene	Rice	n.d.	n.d.	*Xanthomonas oryzae*	(YOSHIMURA *et al.* 1998)
I	*Mi*	2 genes (Mi-1.1 and Mi-1-2), complex gene locus	Tomato	n.d.	n.d.	*Meloidogyne incognita and Macrosiphum euphorbiae*	(MILLIGAN *et al.* 1998; ROSSI *et al.* 1998; VOS *et al.* 1998)
I	*I-2*	1 gene, multigen family	Tomato	n.d.	n.d.	*Fusarium oxysporium*	(SIMONS *et al.* 1998)

II	*L*	13, single gene locus	Flax	yes	*AvrL345*	*Melampsora lini*	(DODDS *et al.* 2006; ELLIS *et al.* 2007; ELLIS *et al.* 1999)
II	*RPP1*	4 genes, multigene family	Arabidopsis	yes	*ATR*^{NdWsB}	*Hyaloperonospora parasitica*	(BOTELLA *et al.* 1998; REHMANY *et al.* 2005)
II	*RPP5*	1 gene, multigene family	Arabidopsis	yes	n.d.	*Peronospora parasitica*	(NOEL *et al.* 1999)
II	*P*	6 genes, multigene family	Flax	yes	*AvrP4*, *AvrP123*	*Melampsora lini*	(CATANZARITI *et al.* 2006; DODDS *et al.* 2001b)
II	*N*	3 genes, complex locus	Flax	yes	n.d.	*Melampsora lini*	(DODDS *et al.* 2001a)
II	*M*	1 gene, complex locus	Flax	n.d.	*AvrM*	*Melampsora lini*	(ANDERSON *et al.* 1997; CATANZARITI *et al.* 2006)
II	*Bs4*	1 gene	Tomato	n.d.	*AvrBs4*	*Xanthomonas campestris*	(KAY *et al.* 2005; SCHORNACK *et al.* 2004)
II	*N*	1 gene	Tobacco	n.d.	*P50*	Tobacco mosaic virus	(UEDA *et al.* 2006; WHITHAM *et al.* 1996)
II	*RPS4*	3, single gene locus	Arabidopsis	n.d.	*AvrRPS4*	*Pseudomonas syringae*	(GASSMANN *et al.* 1999; SOHN *et al.* 2009)
II	*RPP10/ RPP14*	on the same gene locus as RPP1	Arabidopsis	n.d.	n.d.	*Hyaloperonospora parasitica*	(BOTELLA *et al.* 1998)
II	*RPP4*	on the same gene cluster as RPP5	Arabidopsis	n.d.	n.d.	*Peronospora parasitica*	(NOEL *et al.* 1999)
II	*Gro1-4*	1 gene, complex gene family	potato	n.d.	n.d.	*Globodera rostochiensis*	(PAAL *et al.* 2004)
III	*RRS1-R*	1 gene	Arabidopsis	n.d.	*PopP2*	*Ralstonia solanacearum*	(DESLANDES *et al.* 2003)

IV	*Cf2*	1 gene, multigene family	Tomato	n.d.	*Avr2*	*Cladosporium fulvum*	(DIXON *et al.* 1996; LUDERER *et al.* 2002)
IV	*Cf4*	1 gene, multigene family	Tomato	n.d.	*Avr4*	*Cladosporium fulvum*	(JOOSTEN *et al.* 1994; THOMAS *et al.* 1997)
IV	*Cf4A*	1 gene, multigene family	Tomato	n.d.	*Avr4E*	*Cladosporium fulvum*	(TAKKEN *et al.* 1998; WESTERINK *et al.* 2004)
IV	*Cf5*	1 gene	Tomato	n.d.	n.d.	*Cladosporium fulvum*	(DIXON *et al.* 1998)
IV	*Cf9*	1 gene, multigene family	Tomato	n.d.	*Avr9*	*Cladosporium fulvum*	(JONES *et al.* 1994; VANKAN *et al.* 1991)
IV	*Cf9B*	1 gene, multigene family	Tomato	n.d	n.d	*Cladosporium fulvum*	(PANTER *et al.* 2002)
IV	*Hs1blabl*	1 gene, multigene family	Sugar beet	n.d.	n.d.	*Heterodera schacthii*	(CAI *et al.* 1997)

I= CC-NB-LRR, II=TIR-NB-LRR, III= TIR-NB-LRR-WRKY, IV = receptor-like proteins, n.d.= not defined,

pathogen are under diversifying selection (ELLIS *et al.* 2007). Contrasting observations underlying this assumption were made for three R proteins where pathogenic activity is recognised indirectly through a "guard" mechanism. For all three cases, it was demonstrated that they are under balancing and not under diversifying selection (see 1.2.4.).

It remains to be seen if diversifying selection is characteristic for direct interactions, especially as only four cases have been described in which the effector protein of the pathogen is recognized directly: flax *L*/*AvrL567*, rice *Pi-ta*/*AvrPita* and *Arabidopsis RRS1*/*PopP2* and recently, tobacco *N*/*P50* (DESLANDES *et al.* 2003; DODDS *et al.* 2006; JIA *et al.* 2000; UEDA *et al.* 2006). In all four studies, the direct interaction was demonstrated in yeast, and not yet *in vitro*. Thus, it cannot be excluded that a third unknown protein is involved in the obtained interactions between R proteins and their cognate effectors, which is conserved in yeast.

In case of the flax *L* locus, 12 alleles were isolated. The proteins L5 and L6 were found to interact directly by yeast two-hybrid experiments with corresponding flax rust effectors encoded by *AvrL567* genes (DODDS *et al.* 2006). Both, the flax *L* locus as well as the corresponding rust *AvrL567* genes are under diversifying selection (DODDS *et al.* 2006; ELLIS *et al.* 1999).

Rice *Pi-ta* confers resistance against the rice blast disease caused by the fungal pathogen *Magnaporthe grisea* (JIA *et al.* 2000). Its cognate effector gene *AvrPita* is postulated to express a neutral zinc protease (ORBACH *et al.* 2000). Previously, the interaction between Pi-ta and AvrPita was demonstrated in a yeast two-hybrid assay (JIA *et al.* 2000). *Arabidopsis* RRS1 has a C-terminal WRKY domain like some plant transcription factors. Split-ubiquitine analyses in yeast showed that RRS1 interacts directly with PoP2 from *Ralstonia solanacearum* (DESLANDES *et al.* 2003).

Bimolecular fluorescence and co-immunoprecipitation experiments between tobacco N and its cognate effector p50 showed that the TIR domain was involved in the interaction (BURCH-SMITH *et al.* 2007). Additionally, full-length N and p50 interact directly in a yeast two-hybrid assay (UEDA *et al.* 2006). This assay further showed that p50 interacts with a construct that lacks the TIR domain, comprising only the NB-ARC and LRR domains (UEDA *et al.* 2006).

Thus, it seems that the recognition of p50 conferred by N requires all three domains, and that additional plant proteins are required for the interaction that do not exist in the yeast system.
In *Arabidopsis*, the 19 *RPP13* alleles which encode CC-NB-LRR proteins show diversifying selection in their highly polymorphic LRR domains (ROSE *et al.* 2004). Many alleles were also isolated of the corresponding effector *ATR13* from the pathogen *Hyaloperonospora parasitica* (ALLEN *et al.* 2004). So far, it is unknown if RPP13 recognizes its corresponding effectors ATR13 directly or indirectly (MCDOWELL and SIMON 2006). Domain swap analysis between different *ATR13* alleles demonstrated that the encoded C-terminal ATR13 domain triggers RPP13 recognition (ALLEN *et al.* 2008). The interaction between barley *Mla* and powdery mildew AVR_{A10}-$_{K1}$ is proposed to be a third example besides the *RPP13/ATR13* and flax *L/AvrL567* systems to further investigate the hypothesis that the type of biochemical interaction (direct or indirect) correlates with the type of selection pressure (ELLIS *et al.* 2007).

1.2.6. A model for the activation of NB-LRR proteins

Related to the data that demonstrate the importance of the NB-ARC domain for resistance activity of NB-LRR proteins, a model for their activation has been recently proposed (TAKKEN *et al.* 2006). In this model, R proteins fold intramolecularly into an inactive state with bound ADP. This was shown for potato Rx, where the LRR and CC domains interact with the NB-ARC domain (RAIRDAN and MOFFETT 2006). Upon activation by effector recognition, ADP is exchanged with ATP, inducing a molecular switch that results in unfolding of the three domains. The resistance response pathway is induced and finally, ATP hydrolysis restores the resting state (TAKKEN *et al.* 2006). This model is based on studies of the R proteins Mi-1 and I-2 from tomato, for which ATP binding and hydrolysis was demonstrated (TAMELING *et al.* 2002).
Human Apaf-1 (the crystal structure of its NB-ARC domain is shown in Figure 2A) oligomerizes upon activation by cytochrome *c* and ATP to form the apoptosome complex (CAIN *et al.* 2000). So far, tobacco N is the only R protein that was shown to oligomerize, relying on an intact Walker A motif in the NB-ARC domain (MESTRE and BAULCOMBE 2006). The oligomerization of tobacco N suggest the possibility that intramolecular folding and oligomerization upon activation is a widespread characteristic of NB-LRR proteins (VAN OOIJEN *et al.* 2007).

The chaperone HSP90 and the two co-chaperones SGT1 and RAR1 associate and may be involved in R protein stabilization and play an additional role in downstream signalling (SHEN

and SCHULZE-LEFERT 2007). SGT1 is involved in protein degradation through association with components of the SCF ubiquitin ligase complex. Thus, it is possible that the HSP90, SGT1 and RAR1 have a dual role of activation/stabilization and degradation via ubiquitination of R proteins (AZEVEDO *et al.* 2002). Nevertheless, little is known about the machinery that activates R proteins. Silencing experiments revealed the involvement of members of the MAPK signalling cascades and of WRKY transcription factors in tobacco N-induced resistance responses (LIU *et al.* 2004).

Recently, four R proteins were discovered that enter the nucleus: *Arabidopsis* RRS1-R and RPS4, tobacco N and barley MLA10 (BURCH-SMITH *et al.* 2007; DESLANDES *et al.* 2003; SHEN *et al.* 2007; WIRTHMUELLER *et al.* 2007). The first example was *Arabidopsis* RRS1-R, which contains a C-terminal WRKY domain like the WRKY family of transcription factors (DESLANDES *et al.* 2003). RRS1-R directly interacts with its effector PopP2. Fluorescence localization experiments unveiled that RRS1-R nuclear localization depends on the presence of PopP2 (BURCH-SMITH *et al.* 2007; DESLANDES *et al.* 2003; SHEN *et al.* 2007; WIRTHMUELLER *et al.* 2007). The second interesting example is the well-investigated tobacco N (BURCH-SMITH *et al.* 2007). Tobacco N was demonstrated to interact directly with its effector p50 (MESTRE and BAULCOMBE 2006; UEDA *et al.* 2006). Both the R protein N and its effector p50 are localized in both cytosol and nucleus. Strikingly, when tobacco N was fused to a nuclear export signal, no HR was induced in the presence of p50 (BURCH-SMITH *et al.* 2007). The third example is barley MLA10 (see chapter 1.4.2), which interacts through its CC domain with transcription factors HvWRKY1 and HvWRKY2, repressors of the MAMP-induced immune system (SHEN *et al.* 2007). It is suggested that MLA10 unfolds upon activation to establish the interaction with a WRKY transcription factor through its CC domain (SHEN and SCHULZE-LEFERT 2007).

Arabidopsis RPS4 contains a classical nuclear localization signal that allows its import through the nuclear pore complexes. It was shown that nuclear localization is required for the induction of a resistance response. RPS4 triggers a spontaneous and AvrRps4-independent HR when transiently overexpressed in *Nicotiana benthamiana* (ZHANG *et al.* 2004). Expression of RPS4 with a mutated nuclear localization signal abolished the induction of programmed cell death (WIRTHMUELLER *et al.* 2007). Several other R proteins contain a classical nuclear localization signal and are thus proposed to enter the nucleus, e.g. *Arabidopsis* RPM1, RPP1 or RPP13 (see Table 1; CAPLAN *et al.* 2008). Therefore, it is very

likely that most R proteins enter the nucleus to activate the immune response machinery upon effector recognition. It will be very interesting to examine the nuclear localization of R proteins in more detail to shed light on the activation of the recognition response that triggers HR.

1.3 Barley powdery mildew

1.3.1 Fungal life cycle

The barley powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*) is one of the most severe barley diseases. It can be controlled by fungicides or the planting of resistant barley cultivars (SKAMNIOTI *et al.* 2008). Barley powdery mildew is a true obligate biotrophic fungus, obtaining nutrients from the host by haustoria, feeding structures established in a host cell (Figure 3A). The fungus is haploid during most of its life cycle, and the asexually formed conidia are the main source for multiplication and distribution (JORGENSEN 1994). The asexual life cycle starts with the germination of a conidiospore on a barley leaf surface (Figure 3A). After one hour, a primary germ tube develops, followed by the formation of the appressorial germ tube after 12 hours. The appressorium penetrates the host cell for the establishment of the haustorium, which is fully developed after 40 hours. The fungal life cycle is complete after 72 hours by the formation of new conidia spores (PANSTRUGA and SCHULZE-LEFERT 2003). For a short period, the fungus is diploid after sexual fusion between hyphae of opposite mating types to produce the haploid ascospores. This enables crossings between different powdery mildew isolates to genetically investigate fungal genes (SKAMNIOTI *et al.* 2008). Barley powdery mildew is an interesting model organism to study the interactions of a plant host with a biotrophic fungus.

1.3.2. The two effector genes AVR_{A10} and AVR_{K1}

Two powdery mildew effector proteins that are either recognized by *Mla10* or by *Mlk1*, a different powdery mildew resistance gene from barley, were recently isolated from *B. graminis* and designated Avr_{A10} and Avr_{K1} (RIDOUT *et al.* 2006). The alignment of AVR_{A10} and AVR_{K1} shows a sequence identity of only 25% (Figure 3B). So far, the function of the two effectors remains elusive, but it was demonstrated that they belong to a large gene family present in *B. graminis hordei* and *B. graminis formae speciales* (RIDOUT *et al.* 2006). In a cell-death assay, Avr_{A10} and Avr_{K1} were transiently expressed in either barley cultivar Pallas

P09 (*Mla10*) or Pallas P17 (*Mlk*). Thus, the authors demonstrated indirectly that AVR_{A10} may be recognized by MLA10, and AVR_{K1} by MLK (RIDOUT *et al.* 2006).

1.4 The R gene Mla in barley

1.4.1. The host plant barley

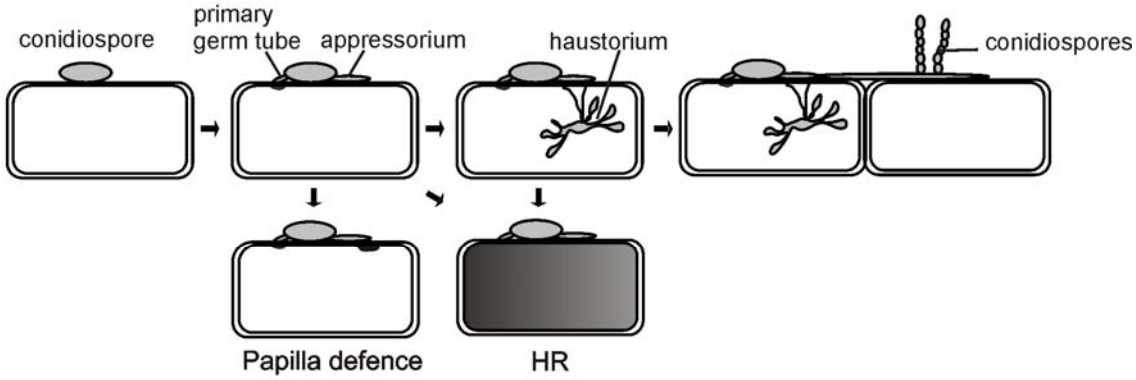
Barley, *Hordeum vulgare* ssp. *vulgare*, is one of the four most important crop plants and mainly used for malting, fodder and human consumption. Barley is a quite adaptive crop plant, and thus, it is cultivated worldwide and under diverse environmental conditions (www.gramene.org). Barley is diploid and self-pollinating, with seven pairs of chromosomes (JORGENSEN 1994). The barley genome is approximately 5000 Mb in size and has not been sequenced yet (MANGELSEN *et al.* 2008). The biotrophic fungus powdery mildew is a common disease for crops like barley. In Europe, barley resistance to powdery mildew has been extensively used in agriculture (JORGENSEN 1994).

1.4.2. The characterisation of Mla resistance

The barley *Mla* locus on chromosome 1H is a quite complex resistance gene locus involved in powdery mildew defence. Its name was derived from "mildew locus a", because the first allele was identified in barley cultivar Algerian (accession number C.I. 1179) and termed *Mla*, later *Mla1* (BROWN and JORGENSEN 1991). More than 30 *Mla* alleles have been genetically described for the *Mla* locus, revealing high functional diversity (DHEERANUPATTANA 1995; JAHOOR and FISCHBECK 1993; JORGENSEN 1994; KINTZIOS *et al.* 1995). The resistance specificities described for all 30 *Mla* alleles were examined by testing the corresponding barley cultivars against different powdery mildew isolates (summarized in DHEERANUPATTANA 1995; JORGENSEN 1994).

Figure 3: Barley powdery mildew and *Mla* resistance responses. A) Asexual life cycle of powdery mildew: the haploid conidiospore first develops a primary germ tube, later an appressorium, which penetrates the host epidermal cell. The haustorium enables the acquisition of nutrients from the host. Finally, the fungus develops new conidiospores. There are two different resistance mechanisms: Either blocking the penetration by cell wall thickening or triggering the HR. B) Amino acid alignment of the two effectors AVR_{A10} and AVR_{K1}. They share a sequence identity of only 25% on the protein level. C) Different *Mla*-induced resistance responses against powdery mildew isolate K1 seven days after infection scored according the following five macroscopic infection types (ITs): (*i*) Barley cultivar Pallas P01 is fully resistant. (*ii*) Ingrid cultivar IGV3-003 triggers HR visible as necrotic spots. (*iii*) Ingrid cultivar IGV3-002 shows intermediate resistance, as several colonies develop until sporulation before HR is triggered. (*iv*) Colonies with slight necrotic spots are observed for Ingrid cultivar IGV3-097 and (*v*) Pallas P03 is fully susceptible.

A



B

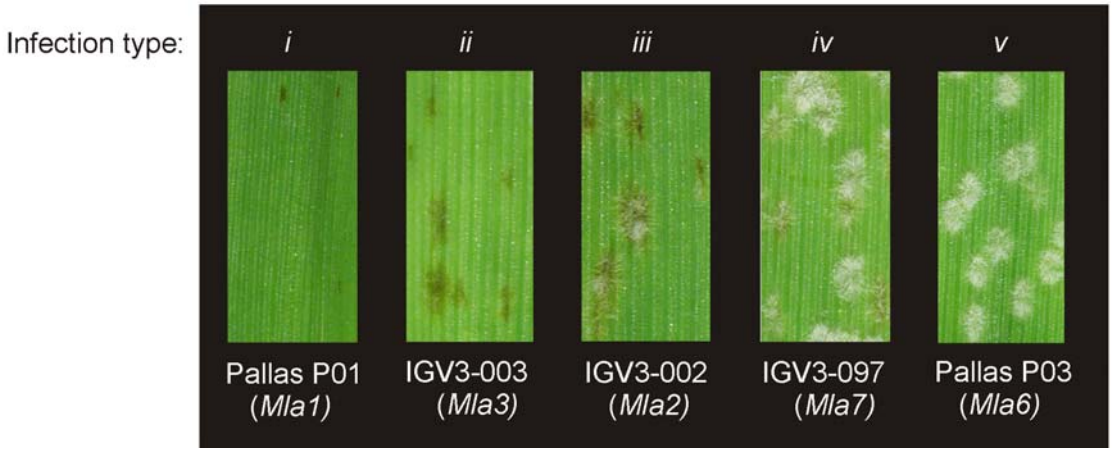
```
AVRA10 MDSSHSKPREKHANKKRTRVQSSKYEHHRGCAPSEGERCPRGRYGGRRSREAKNPRRFNFGILQPRRCRASLG
AVRK1  ------------------------------------------------------------------------MIG

AVRA10 SHRIPQKTVVAPEKRAAVPALPRPVEVRPTKEADI-PTAKAAGAENSSLDQPPTAPEAAGREKIF--PPELLA
AVRK1  SVEII-------EKISQLPSVPHRIGELSKSP----PTASKRS-ENAAPISAPKLMSQLKAATKAECPPELRP

AVRA10 AIEAEERRARQKAAQFTICSTAISSVEAALQPLSTGEDKNFVDSIKVYLRAAIAQFVAAGESTAPPVLPQRPS
AVRK1  IVEAIQQRAAKTAANLALCSAAISGVEATLLPLTNGSNRPFVDSMRVYLRAAIAQYIATVEASTPPVLPPRLA

AVRA10 VGTPATVIPVAIPRNISKATAPLPLRSTWATVTRAGHQKSGAQAPANITSVSAAPSARKQEKKPAAATTA
AVRK1  NPLPRAPDARSIQIPAVP--ALPLKSTRATVVKMDCHKKQFL----------------------------
```

C



The visible resistance responses vary for different *Mla* alleles. The observed host responses against the two powdery mildew isolates A6 and K1 were classified into five macroscopic infection types (ITs). Some *Mla* alleles are able to interfere with fungal penetration by inducing the formation of papillae, thickenings of the cell wall (Figure 3A). Such a resistance response is judged as an IT of *i* (Figure 3C). The classical *R* gene defence mechanism to prevent the fungus from getting nutrients is the HR. This programmed cell death visualizes as brown spots on the leaf surface and is assessed as a resistance phenotype of *ii* (Figure 3C). Finally, there are several intermediate phenotypes, from inhibition of fungal growth (IT of *iii* - *iv*) to complete susceptibility (IT of *v*), where the fungus is completely able to develop until sporulation. The timing and the distribution of HR for different *Mla* alleles was examined previously. *Mla3* and *Mla7* were shown to stop fungal growth at a later stage of the infection process whereas *Mla1* and *Mla6* terminated the infection early (BOYD *et al.* 1995)**.**

Six *Mla* alleles (*Mla1, Mla6, Mla7, Mla10, Mla12 and Mla13*) have already been isolated (HALTERMAN *et al.* 2001; HALTERMAN *et al.* 2003; HALTERMAN and WISE 2004; SHEN *et al.* 2003; ZHOU *et al.* 2001). They encode CC-NB-LRR type of R proteins that share sequence identities of more than 90%. The sequencing of 261 kb at the *Mla* locus in the susceptible barley cultivar Morex revealed eight CC-NB-LRR resistance gene homologues (*RGH*s) that belong to three unrelated families, designated *RGH1*, *RGH2* and *RGH3*(WEI *et al.* 2002). The *RGH1bcd* gene disrupted by a *BARE-1* transposon showed the highest similarity of about 80% to the cloned *Mla* alleles on the nucleotide and amino acid levels (WEI *et al.* 2002).

The chaperone HSP90 and the two co-chaperones RAR1 and SGT1 are important for effective resistance responses induced by MLA (BIERI *et al.* 2004). RAR1 encodes a zinc binding protein, whereas SGT1 is involved in the ubiquitin pathway and therefore assumed to be important for the regulation of both R protein stability and degradation (BOTER *et al.* 2007). Domain swap analysis between MLA1 and MLA6 revealed that the LRR domain is involved in recognition specificity. A chimera between the CC-NB-ARC domain of MLA6 and the LRR domain of MLA1 gave the same strong resistance response against powdery mildew K1 as did full-length MLA1 (SHEN *et al.* 2003). It is still unknown if MLA confers recognition by direct or indirect interaction with its cognate effector molecule. Recently, MLA10 was demonstrated to localize not only in the cytosol, but also in the nucleus. Furthermore, the first 46 residues of MLA10 interact with the two transcription factors HvWRKY1 and HvWRKY2. HvWRKY1 and HvWRKY2 were shown to act as repressors of basal defence mechanisms

(SHEN *et al.* 2007). It is assumed that MLA is activated upon recognition of its cognate effector through an unknown mechanism. Upon activation, MLA10 enters the nucleus and interacts with HvWRKY1/HvWRKY2, which leads to their inhibition and derepression of defence mechanisms (SHEN and SCHULZE-LEFERT 2007).

1.4.3. Other powdery mildew genes in barley

Five additional gene loci were mapped on barley chromosome 1H that are involved in resistance against powdery mildew: *Mlat*, *MlGa*, *Mlnn*, *Mlra* and *Mlk* (JORGENSEN 1994). It was shown previously that *Mlk* is closely linked to the *Mla* locus (GIESE *et al.* 1981). So far, none of them has been cloned and further characterised. Besides *Mla*, there is only a second resistance gene, called *Mlo*, that has been isolated. The lack of *Mlo* in corresponding mutants confers a durable broad-spectrum powdery mildew resistance. Thus, *Mlo*-lacking plants have become a very important source of barley powdery mildew resistance in Europe (JORGENSEN 1994). MLO is predicted to be a seven-transmembrane protein, and it may play a role in vesicle trafficking involved in defence. It was demonstrated that MLO is required for the entry of powdery mildew species into the monocot barley as well as into the dicot *Arabidopsis,* suggesting a conserved mechanism for entry into host cells (CONSONNI *et al.* 2006).

1.5. Aims of this thesis

The characterisation of the barley germplasm for *Mla* specific-resistance against powdery mildew revealed a polymorphic *Mla* gene locus (JORGENSEN 1994). More than 30 barley cultivars were found to harbour distinct *Mla* alleles in classical genetic studies. Previously, only six *Mla* alleles have been isolated and characterised at the molecular level (*Mla1*, *Mla6*, *Mla10*, *Mla12* and *Mla13*). The availability of these well-described and diverse barley cultivars set the ground for our molecular investigation of the *R* gene *Mla* and the isolation of new *Mla* variants.

We wanted to investigate how polymorphic the *Mla* locus is by cloning and characterising new *Mla* sequences designated as candidate MLA cDNAs. Furthermore, we intended to determine in a transient overexpression assay if the newly isolated candidate MLA cDNAs give a resistance response when tested against the two isolates A6 and K1. As the LRR domain is highly diverse for the six known MLA proteins, it possibly determines recognition specificity. If similar sequence variation would be detected in the newly isolated candidate MLA cDNAs, the diversity of MLA would help to precisely identify the sequence requirements for the detection of the cognate pathogenic effectors. Thus, we also wanted to test the feasibility of an assay to efficiently test validated and candidate MLA cDNAs against powdery mildew effector proteins.

2. Diversity of isolated validated and candidate MLA cDNAs

2.1 Introduction

Plants have a basal defence system similar to mammals, consisting of membrane associated pattern-recognition receptors (PRRs) that detect evolutionarily conserved molecules produced by pathogens called MAMPs (microbe-associated molecular patterns; SHEN and SCHULZE-LEFERT 2007). A second, adaptive defence system specific for plants consists of resistance (*R*) genes. The majority of these *R* genes encode intracellular proteins of the NB-LRR (nucleotide binding and leucine-rich repeat) type (SHEN *et al.* 2007). They are classified according to their N-terminal domain in TIR-NB-LRR (Toll-like) and CC-NB-LRR (coiled-coil) type of proteins (DANGL and JONES 2001). These R proteins can detect pathogen-derived effector molecules either directly or indirectly and hence trigger hypersensitive response (HR), a fast cell death of the infected host cell, through a mostly unknown mechanism (JONES and DANGL 2006).

LRR domains encoded by resistance genes are thought to determine recognition specificity (DEYOUNG and INNES 2006). This assumption is based on studies about the involvement of LRR domains of several protein families like the mammalian Toll-like receptors in ligand binding or protein-protein interactions (BENT and MACKEY 2007; LIEW *et al.* 2005). A typical leucine-rich repeat consists of 20 to 29 residues that form a β strand followed by an α helix that are arranged parallel to a common axis (KAJAVA *et al.* 1995). The conserved segment of 12 amino acids (LxxLxLxxN/CxL) of the β-strand/β-turn motif lies on the solvent-exposed, concave face, where x stands for any residue and the L position can be occupied by all hydrophobic amino acids (KOBE and KAJAVA 2001). The leucine or related residues of the LRR motif point inward and build a hydrophobic core that gives a lateral stabilization to the architecture as it was deduced from the crystal structure of human Toll-like receptor TLR3 (CHOE *et al.* 2005). The typical α-helix is described as the convex face of the LRR.

Several studies revealed that residues of the solvent-exposed LxxLxLxx β-sheet motif in the LRR domain are under stronger positive selection than other parts of the proteins (DUNNING *et al.* 2007). In particular, diversifying selection was shown for NB-LRR genes with allelic series like *Arabidopsis RPP13*, the flax *L* gene, or wheat *Pm3* as nonsynonymous substitutions are overrepresented in comparison to changes in synonymous sites (ELLIS *et al.*

1999; ROSE *et al.* 2004; YAHIAOUI *et al.* 2006). It was recently proposed that in cases of direct recognition, both the *R* gene and the corresponding gene of the pathogen are under diversifying selection (ELLIS *et al.* 2007). Contrasting observations underlying this assumption were made for two cases where pathogenic activity is recognized indirectly through a "guard" mechanism. The *Arabidopsis* genes *RPM1* and *RPS5* encoding "guardee" proteins were shown to be conserved and under balancing selection (BENT and MACKEY 2007; STAHL *et al.* 1999; TIAN *et al.* 2002). It remains to be seen if diversifying selection is characteristic for direct interactions as only four cases have been described in which the effector protein of the pathogen is recognized directly: flax *L*/*AvrL567*, tobacco N/p50, rice *Pi-ta*/*AvrPita* and *Arabidopsis RRS1*/*PopP2 (DESLANDES et al. 2003; DODDS et al. 2006; JIA et al. 2000; UEDA et al. 2006)*. In case of the flax *L* locus, 12 alleles were isolated. The proteins L5 and L6 interacted directly in yeast-two-hybrid experiments with corresponding flax rust effectors encoded by *AvrL567* genes (DODDS *et al.* 2006). Both, the flax *L* locus as well as the corresponding rust *AvrL567* genes are under diversifying selection (DODDS *et al.* 2006; ELLIS *et al.* 1999).

In *Arabidopsis*, the 19 *RPP13* alleles which encode CC-NB-LRR proteins show diversifying selection in their highly polymorphic LRR domains (ROSE *et al.* 2004). Many alleles were also isolated from the corresponding effector *ATR13* of the pathogen *Hyaloperonospora parasitica* (ALLEN *et al.* 2004). So far, It is unknown if RPP13 recognizes its corresponding effectors ATR13 directly (MCDOWELL and SIMON 2006).

The barley-powdery mildew *Mla/AVR$_{A10}$* interaction is proposed to be a third example besides the flax *L/AvrL567* and *RPP13/ATR13* systems to investigate further the hypothesis about the correlation of the type of biochemical interaction (direct or indirect) and selection pressure (ELLIS *et al.* 2007). The characterisation of resistance specificity against different powdery mildew (*Blumeria graminis* f. sp. *hordei*) isolates in barley (*Hordeum vulgare* ssp. *vulgare*), revealed high functional diversity at the *Mla* locus (JORGENSEN 1994). The availability of this well-described and diverse germplasm was the basis for our molecular investigation of the *R* gene *Mla* and the isolation of new alleles. More than 30 *Mla* alleles have been genetically described for the *Mla* locus on barley chromosome 1H (DHEERANUPATTANA 1995; JAHOOR and FISCHBECK 1993; JORGENSEN 1994; KINTZIOS *et al.* 1995). Six *Mla* alleles (*Mla1, Mla6, Mla7, Mla10, Mla12 and Mla13*) have already been isolated (HALTERMAN *et al.* 2001; HALTERMAN *et al.* 2003; HALTERMAN and WISE 2004; SHEN *et al.* 2003; ZHOU *et al.* 2001).

They encode CC-NB-LRR type of R proteins that share sequence identities of more than 90%. Sequencing of 261kb at the *Mla* locus in the susceptible barley cultivar Morex revealed eight CC-NB-LRR resistance gene homologues (*RGH*s) that belong to three unrelated families designated *RGH1*, *RGH2* and *RGH3* (WEI *et al.* 2002). The *RGH1bcd* gene hosting a *BARE-1* transposon showed the highest similarity of about 80% to the cloned *Mla* alleles on nucleotide and amino acid level (WEI *et al.* 2002).

Two powdery mildew effector proteins that are either recognized by *Mla10* or by *Mlk1*, a different powdery mildew resistance gene from barley, were recently isolated from *B. graminis* and designated AVR_{A10} and AVR_{K1} (RIDOUT *et al.* 2006). So far, the protein's function of these two effector genes remains elusive, but it was demonstrated that they belong to a large gene family present in *B. graminis hordei* and *B. graminis formae speciales* (RIDOUT *et al.* 2006).

Here, we describe the molecular isolation and analysis of 23 new *Mla* genes designated as candidate MLA cDNAs. The analysis of the complete set of 29 MLA cDNAs revealed a high level of polymorphism in the LRR domain with a strong, gradient increase in the C-terminal direction. Additionally, our data show that *Mla* is under diversifying selection. From 34 residues that are under positive selection, 18 lie in the LxxLxLxx motif on the solvent-exposed side of the LRR domain.

2.2 Results

2.2.1. Diversity of infection phenotypes in barley cultivars containing different *Mla* specificities

Six *Mla* resistance alleles, *Mla1*, *Mla6*, *Mla7*, *Mla10*, *Mla12* and *Mla13* were previously isolated (HALTERMAN *et al.* 2001; HALTERMAN *et al.* 2003; HALTERMAN and WISE 2004; SHEN *et al.* 2003; ZHOU *et al.* 2001). In this study, we aimed to isolate further resistance alleles using barley accessions that contain different *Mla* resistance specificities, as inferred from differential resistance responses to a set of *B. g.* f sp *hordei* isolates and genetic linkage to the *Mla* locus on barley chromosome 1H (see Table 2; JORGENSEN 1994). Many of the previously used powdery mildew isolates are no longer available. We therefore tested these barley accessions for differential infection phenotypes to two powdery mildew isolates, A6 and K1, which have been shown to contain at least seven and five AVR_A effectors,

respectively (see chapter 2.4.1.; BIERI *et al.* 2004). Barley leaves were inoculated with fungal conidiospores and examined after seven days for disease resistance against the two isolates. The observed host responses were classified into five macroscopic infection types (ITs; Table 2). The survey revealed in 18 of the 43 accessions isolate-specific ITs (Table 2), indicating the presence of several additional AVR_A or other *AVR* genes in the A6 or K1 strain. Growth of both fungal strains was severely restricted in 15 accessions (IT 2 or lower, Table 2), suggesting these resistance responses are triggered by AVR_A or other *AVR* genes that are shared by the A6 and K1 isolates. The large number of additional putative AVR_A genes in A6 and K1 prompted us to assess the functions of candidate MLA cDNAs in a transient gene expression assay following challenge with these isolates (see below).

Table 2: Barley cultivars used for the isolation of validated and candidate MLA cDNAs and their infection phenotypes to powdery mildew isolates A6 and K1.

The observed host responses against the two powdery mildew isolates A6 and K1 were scored seven days post inoculation and classified into five infection types (ITs): (*i*) full immunity without detectable fungal growth, (*ii*) barely detectable fungal growth typically surrounded by necrotic patches, (*iii*) occasional fungal growth surrounded by necrotic patches, (*iv*) extensive fungal growth and sporulation with few necrotic patches, and (*v*) profuse fungal growth and sporulation without detectable necrotic areas. Isolated candidate MLA cDNAs labeled with an asterisk (*) were confirmed to be active resistance genes with at least one of the fungal isolates A6 and K1 in the transient assay (see Figure 6). n.d. = not detected. [a]It was suggested that the near-isogenic Ingrid line of Franger harbours the specificity *Mla6* instead of *Mla14 (JORGENSEN 1994)*, which is confirmed by the isolation of *Mla6* in this work. [b] It was suggested that Long Glumes harbours the specificity *Mla7* instead of *Mla15* (KINTZIOS *et al.* 1995), which is confirmed by the isolation of *Mla7* in this work. [c](JAHOOR and FISCHBECK 1987). [d] It was suggested that Engledow India harbours specificity *Mla13* instead of *Mla24* (JORGENSEN 1994), which was confirmed by the isolation of *Mla13* in this work. [e](JAHOOR and FISCHBECK 1993), [f](KINTZIOS *et al.* 1995), [g](DHEERANUPATTANA 1995).

Barley Cultivars		***Mla* resistance specificities**		**scored infection phenotypes**		
Name	**Identifier**	**proposed specificities**	**cDNA identifier**	**A6**	**K1**	**Reference**
Iso 1R Manchuria isogenic (Algerian, *Mla1*)	CIho 16137	1	1	*v*	*i*	ZHOU *et al.* 2001
Ingrid isogenic (Black Russian, *Mla2*)	IGV 3-002	2	2*	*iv*	*iii*	this work
Ingrid isogenic (Ricardo, *Mla3*)	IGV 3-003	3	3*	*ii*	*ii*	this work
Gopal	PI 41162	5	7	*iii*	*iii*	this work
Iso 20R Manchuria isogenic (Franger, *Mla6*)	CIho 16151	6	6	*i*	*v*	HALTERMAN *et al.* 2001
Iso 10R Manchuria isogenic (Multan, *Mla7*)	CIho 16147	7	7	*iii*	*iv*	HALTERMAN and WISE 2004
Golden Promise	PI 467829	8	8*	*v*	*v*	this work
Ingrid isogenic (Akka, *Mla9*)	IGV 3-009	9	9*	*ii*	*v*	this work
Iso 12R Manuchuria isogenic (Durani, *Mla10*)	CIho 16149	10	10	*i*	*v*	HALTERMAN and WISE 2004
Ingrid isogenic (A222, *Mla11*)	IGV 3-011	11	8	*v*	*v*	this work
Ingrid isogenic (Emir, *Mla12*)	IGV 3-012	12	12	*ii*	*v*	SHEN *et al.* 2003
Manchuria isogenic (Rupee, *Mla13*)	CIho 16155	13	13	*i*	*i*	HALTERMAN *et al.* 2003
Ingrid isogenic (Franger, *Mla14*)[a]	IGV 3-015	14	6	*i*	*v*	this work
Long Glumes [b]	CIho 6168	15	7	*ii*	*v*	this work
Diamant x 1B-54B[c]		16	16-1	*i*	*i*	this work
RS 170-47[c]		17	n.d.	*ii*	*i*	this work
RS20-1 x Kiebitz B[c]		18	18-1	*i*	*i*	this work
RS20-1 x Kiebitz B[c]		18	18-2*	*i*	*i*	this work
Diamant x 1B-86B[c]		19	19-1	*i*	*i*	this work
RS145-39 x Kiebitz[c]		20	25-1	*i*	*i*	this work
Ingrid isogenic (Hordeum 1657, *Mla22*)	IGV 3-067	22	22*	*v*	*i*	this work
Ingrid isogenic (Hordeum 1402, *Mla23*)	IGV 3-066	23	23*	*iii*	*iii*	this work
Engledow India[d]	Clho 7555	24	13	*i*	*i*	this work
RS170-10 x Piccolo A[e]		25	25-1	*ii*	*ii*	this work
Diamant x 1B-20[e]		26	n.d.	*ii*	*i*	this work
RS1-8 x Piccolo E[e]		27	27-1*	*ii*	*i*	this work
RS1-8 x Piccolo E[e]		27	27-2*	*ii*	*i*	this work
Diamant x 1B-151[e]		28	28*	*i*	*i*	this work
110-4 x Sonja[f]		29	n.d.	*iv*	*ii*	this work
Nigrate	HOR 14775	30	30-1	*iii*	*v*	this work
Turkey 290	CIho 13646	31	31-1	*v*	*v*	this work
142-29 x Dura[f]		32	32*	*i*	*i*	this work
RS70-29[g]		34	34*	*i*	*i*	this work
Sv. 57/510-44	CIho 14013	MlTu2	35-1*	*i*	*i*	this work
Arlington Awnless	Clho 702	Mly	36-1	*iii*	*v*	this work
Kairyobozu-mugi	CIho 11556	Mlkb	37-1	*v*	*i*	this work
Nepal	PI 12709	Mln	38-1	*iv*	*iv*	this work
Nigri Nudum	CIho 11549	Mlnn	39-1	*iv*	*iv*	this work
Nakaizumi-Zairai	CIho 11561	Mlk2	2	*iv*	*iii*	this work
Vogelsanger Gold	PI 406266	Mlra	6	*i*	*iv*	this work
Atlas	ChI 13824	Mlat	8	*v*	*iv*	this work
Magnif 105	PI 337142	Mla_{ma}	8	*iii*	*iv*	this work
52	PI 134257	Mlci	8	*v*	*v*	this work
Marco	PI 94877	MlMa	31	*iv*	*v*	this work
Russian 81	Clho 11546	Mlr81	39-1	*iv*	*ii*	this work

2.2.2. A cDNA-based PCR approach to isolate *Mla* sequences

We developed a cDNA-based PCR approach to isolate candidate MLA cDNAs. By this means we excluded the isolation of potentially non-expressed *Mla* homologues from the barley genome. A similar cDNA approach was used before to isolate *Mla7* and *Mla10* (HALTERMAN and WISE 2004). Among the six previously isolated *Mla* alleles, the 3'-region encoding the LRRs is highly diverse and contains interspersed short stretches of conserved sequences. In contrast, the 5'-region, including the 5'-untranslated region (UTR), and the sequence encoding the CC domain is well conserved. This sequence conservation pattern instigated the development of a two-step protocol to isolate candidate MLA cDNAs. First, PCR primers were designed from short conserved parts in the 3'region encoding the LRR for 3'-RACE, typically resulting in approximately 700 bp fragments. If this 3'-RACE did not produce amplicons, we employed a 3'-UTR PCR using primers in conserved stretches upstream and downstream of the stop codon (see chapter 2.4.2.). After sequencing of the amplicons, the sequence information was used in a second step to design a specific PCR downstream of the stop codon to amplify the complete coding region of candidate MLA cDNAs. Two specific primers within the 5'-UTR were then designed and used to amplify the entire approximately 3.0 kb long fragment of 19 candidate MLA cDNAs (see chapter 6.6). Of these, 15 were isolated from barley accessions believed to harbour different *Mla* resistance specificities and the cDNAs were named accordingly (JORGENSEN 1994). Four additional candidate MLA cDNAs were obtained using a 5'-UTR primer derived from the *Hordeum chilense HcMla1* homologue (unpublished data). Collectively, these experiments resulted in the isolation of 23 candidate MLA cDNAs whose complete DNA sequences were determined (Table 2). Out of a total of 37 accessions examined, we failed to obtain cDNA amplicons from three lines (containing Mla17, Mla26, or Mla29; JORGENSEN 1994). This might be due to nucleotide polymorphisms at PCR primer annealing sites in the corresponding MLA cDNAs.

We recovered five candidate MLA cDNAs from accessions for which it was previously unclear whether race-specific immunity is conferred by the *Mla* or a different *R* locus on chromosome 1H (JORGENSEN 1993; JORGENSEN 1994). Because these cDNAs were recovered from accessions that do not belong to the original set of barley lines defining 34 *Mla* resistance specificities (JORGENSEN 1994), but, nevertheless, show extensive sequence relatedness to known *Mla R* genes, we named the corresponding genes *Mla35-1*, *Mla36-1*, *Mla37-1* and *Mla39-1* (Table 2). This nomenclature was adopted in analogy to previous studies (HALTERMAN *et al.* 2003; ZHOU *et al.* 2001) to denote that these genes might be non-

functional and/or could be *MLA* homologues linked or unlinked to *MLA*. The MLA38-1 cDNA was isolated from the barley landrace Nepal and contains an in-frame stop codon that predicts a severely truncated protein of 232 amino acids (Figure 4). Since identical cDNA sequences were obtained from two independent cDNA synthesis reactions, it is likely that *Mla38-1* encodes a non-functional MLA homologue. We thus excluded this cDNA from further computational analysis (see below). Unexpectedly, we isolated from some barley accessions cDNAs that were identical to those described before. Cultivar Gopal has been described to contain *Mla5* (JORGENSEN 1994), but the cDNA isolated from this accession is identical with the known MLA7 cDNA (HALTERMAN and WISE 2004). Similarly, we isolated from the near-isogenic *Mla11* backcross line Ingrid (A222, *Mla11*) cDNAs that were identical with those identified in cultivar Golden Promise containing *Mla8*. Finally, the MLA25-1 cDNA was independently isolated twice from accessions RS170-10 x Piccolo A and RS145-39 x Kiebitz B. The latter accession was described to contain *Mla20* (Table 2; JAHOOR and FISCHBECK 1987). These discrepancies are most likely the result of accidental seed contamination. We excluded gene products encoded by these duplicate cDNA sequences from the graphic protein alignment shown in Figure 4.

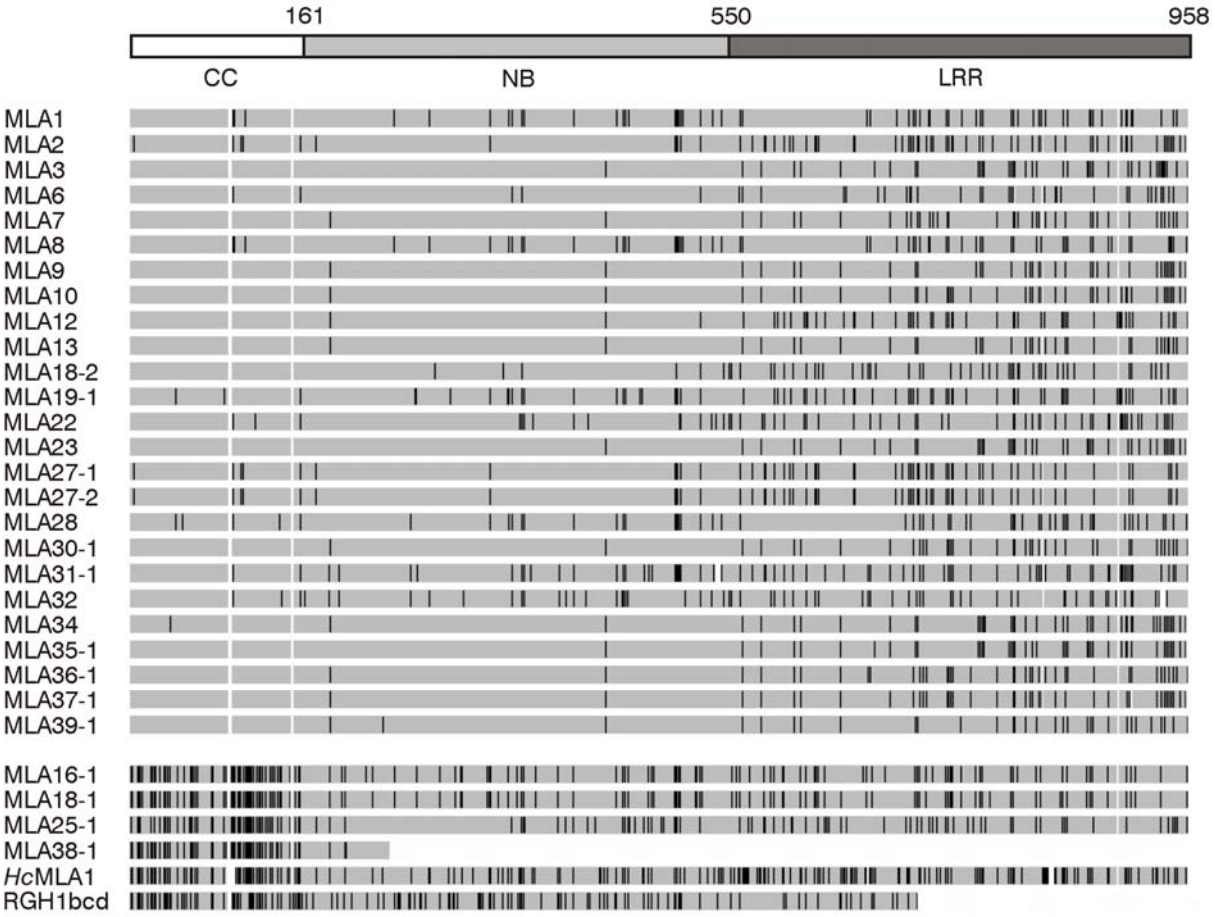


Figure 4: Polymorphic sites cluster in the LRR of candidate and validated MLA resistance specificities. The proteins encoded by 29 validated and candidate MLA cDNAs are schematically represented by a consensus sequence (grey). Polymorphic residues are illustrated in black. The protein sequence deduced from *Mla38-1* is truncated due to a stop codon at position 233. Two additional protein sequences encoded by *Mla* homologs were included: The 282 amino acid-long sequence of RGH1bcd was obtained from barley cultivar Morex (WEI *et al.* 2002) and *Hc*MLA1 from *H. chilense*. The three domains of MLA are indicated at the top. White: gap in the sequence alignment.

2.2.3. Few barley lines contain more than one candidate MLA cDNA

Unexpectedly, we isolated from RNA of two cultivars in each case two different candidate MLA cDNAs. Cultivar RS1-8 x Piccolo E was found to harbour candidate cDNAs that differ from each other only close to the 3'end. In accordance with the nomenclature mentioned above, we designated these transcripts MLA27-1 and MLA27-2. MLA27-1 is six amino acid residues shorter than MLA27-2 and differs in three of the last four residues from MLA27-2. In contrast, cultivar RS20-1 x Kiebitz B was found to contain two transcripts with much sequence divergence throughout their lengths, predicting proteins sharing only 82% similarity. These cDNAs were designated MLA18-1 and MLA18-2. The detection of two candidate MLA cDNAs in *Mla18* and *Mla27* containing plants was unexpected as the Southern blot hybridization patterns of their DNA with a *Mla1* probe was similar to other lines from which only one cDNA species was isolated (Figure 5; see also below). To exclude the possibility that the detection of two candidate cDNAs was the result of heterozygosity at the *Mla* locus, we self-fertilized single plants of the respective cultivars for three subsequent generations. Re-isolation of the same two candidate cDNAs from selfed S_3 individuals (not shown) strongly suggests these are derived from *Mla* paralogs.

Sequence alignment of gene products encoded by the candidate cDNAs with the six known MLA proteins (MLA1, MLA6, MLA7, MLA10, MLA12 and MLA13) revealed a polymorphic LRR, and, with four exceptions, a highly conserved CC domain (Figure 4). The first 46 residues of the CC domain are sufficient to interact with the transcription factors *Hv*WRKY1 and *Hv*WRKY2 (SHEN *et al.* 2007) and within this stretch 22 amino acids (residue 26 to 47) are predicted to adopt a coiled-coil structure (ZHOU *et al.* 2001). This 22 amino acid long coiled-coil was detected in all 29 protein models using the program COILS (LUPAS *et al.* 1991). Within the N-terminal 46 residues only three amino acid polymorphisms were found among 25 of the total of 29 deduced proteins. Notably, the remaining four candidate cDNAs (Mla16-1, MLA18-1, MLA25-1, and MLA38-1), each encoding unusually

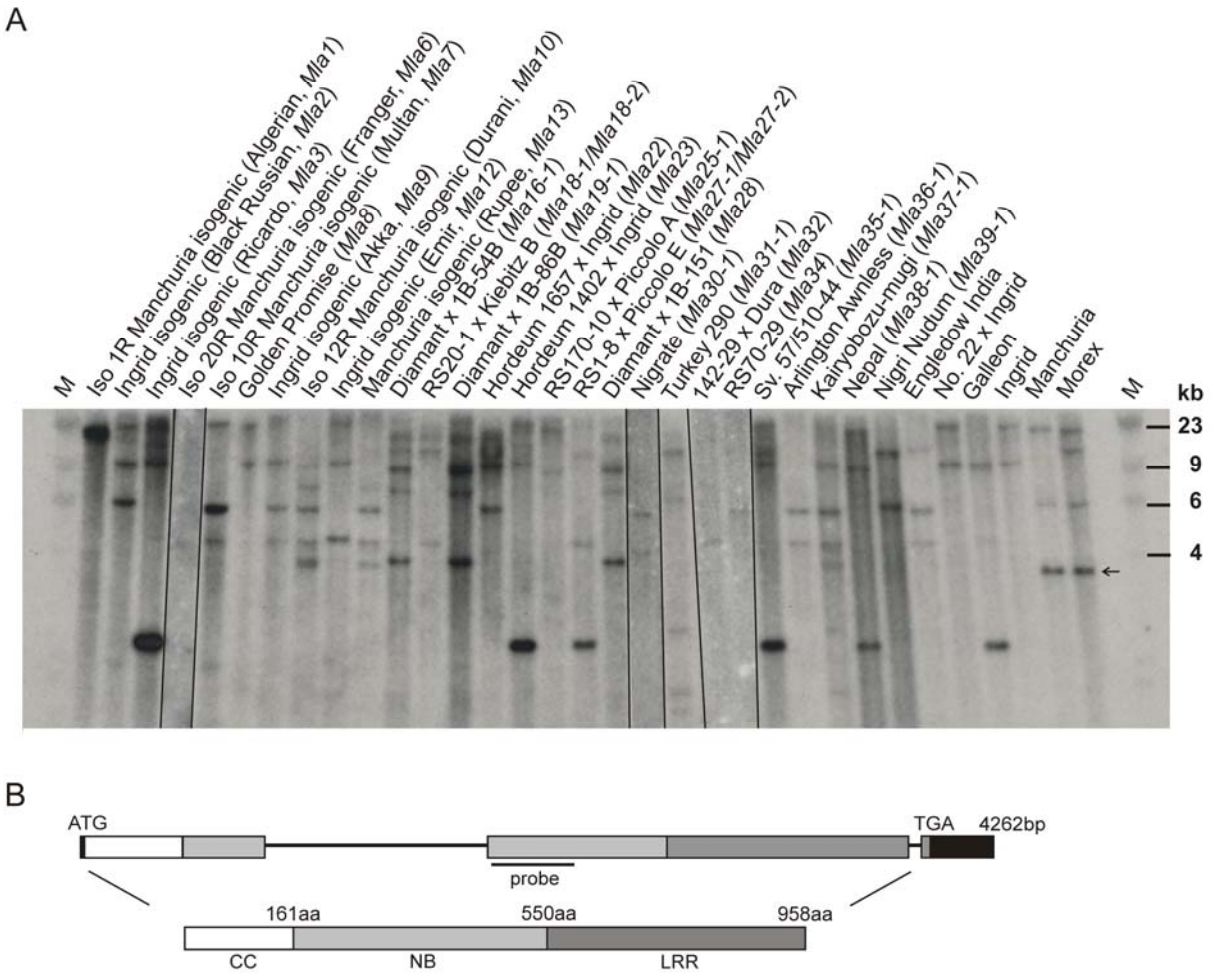


Figure 5: Diversity survey of *Mla* homologs. A) Southern blot analysis. Genomic DNA was isolated from the indicated 29 barley accessions/cultivars from which the candidate and validated MLA cDNAs were isolated, and was digested with *Eco*RI. The autoradiography shown is composed of two independent Southern blot experiments with qualitatively identical results. The indicated 501 bp DNA fragment (in B) was used as a probe and was amplified from the region encoding the NB domain of *Mla1*. For cultivar Morex, four hybridization signals are visible. The lowest corresponds to *RGH1bcd* (arrow) and the other signals to the *Mla* homologs *RGH1a, RGHe*, and *RGHf*. The truncated gene *RGH1bcd* is proposed to be a non-functional *Mla* allele (WEI *et al.* 2002). Most barley accessions show a hybridization pattern with fewer bands than Morex. M: size marker. B) The genomic region of *Mla1* (4262 bp) and the corresponding protein are depicted. The region amplified for the hybridization probe is indicated.

polymorphic CC domains, are most similar to the deduced N-terminus of *RGH1bcd* in cultivar Morex (WEI *et al.* 2002). Morex lacks a detectable *Mla* resistance specificity and *RGH1bcd*, encoding a truncated protein, is considered as non-functional *Mla* allele (WEI *et al.* 2002).

To assess the genomic diversity of the *Mla* locus in the examined barley cultivars/accessions, we performed a Southern blot analysis using a 501 bp *Mla1* probe, which encodes the C-terminal end of the NB-ARC domain (Figure 5). Consistent with a previous report, four hybridization signals were detected in DNA from cultivar Morex that correspond to four resistance gene homologues (*RGHs*) at the *Mla* locus, designated *RGH1a, RGH1bcd, RGH1e* and *RGH1f* (Figure 5; WEI *et al.* 2002). Although we detected in this diversity survey several distinct hybridization patterns between accessions, most accessions showed fewer and the remaining lines a similar number (up to six) of hybridization signals in comparison to Morex (Figure 5). This indicates limited variation in the number of *Mla* paralogs and the lack of unusual paralog expansion in the examined germplasm.

2.2.4. Functional analysis of candidate MLA cDNAs

We used a transient gene expression assay to assess the disease resistance activity of the candidate MLA cDNAs following particle-mediated delivery of plasmid DNA in single barley leaf epidermal cells (SHEN *et al.* 2003). Expression of the cDNAs is driven by the strong ubiquitin promoter and disease resistance was quantitatively scored as powdery mildew haustorium index (%) in epidermal cells attacked by sporelings of A6 or K1 isolates two days after conidiospore inoculation (SHEN *et al.* 2003). Of 23 tested candidate *Mla* cDNAs, 13 significantly restricted haustorium development (Figure 6). Eight alleles showed a strong resistance response (low haustorium index from 5% to 40%) and five alleles showed an intermediate resistance of 50% to 70% that was statistically different ($P \leq 0.05$) from the susceptible control. MLA27-1 and MLA27-2 were tested against both isolates (K1 and A6) and each reduced the haustorium index to a similar level. The latter finding strongly suggests that cultivar RS1-8 x Piccolo E, containing the presumed paralogs *Mla27-1* and *MLA27-2*, harbours two functional *Mla* genes.

We failed to detect significant disease resistance activity upon expression of candidate cDNAs MLA16-1, MLA18-1, MLA19-1, MLA25-1, MLA30-1, MLA31-1, MLA36-1, MLA37-1, MLA39-1, and, expectedly, MLA38-1, which encodes a truncated protein of 232

amino acids (not shown). Undetectable disease resistance activity upon transient gene expression of MLA30-1, MLA31-1, and MLA39-1 is consistent with unrestricted growth of A6 and K1 isolates on the corresponding accessions Nigrate, Turkey 290, and Nigri Nudum (each ITs iii - iv; Table 2). However, undetectable disease resistance activity of candidate cDNAs MLA16-1, MLA19-1, and MLA25-1 in the transient gene expression assay contrasts with fully resistant phenotypes in the corresponding lines Diamant x 1B-54B, Diamant x 1B-86B, and RS170-10 x Piccolo A (Table 2). Similarly, cultivar Kairyobozu-mugi (*Mla37-1*) exhibited isolate-specific full immunity to the K1 fungus (Table 2), but the isolated candidate MLA37-1 cDNA conferred no resistance activity in the transient gene expression experiments (not shown). It is thus possible that the disease resistance responses in these lines are conferred by a *R* gene other than *Mla* or that the isolated candidate cDNAs are derived from *Mla* homologues that do not detect A6 and K1 effectors.

Candidate cDNAs Mla16-1, MLA18-1, MLA25-1 as well as MLA38-1 each encode exceptionally polymorphic CC domains compared to other validated or candidate MLAs (Figure 4; for details see chapter 3). Each of these four CC domains is most closely related to the CC encoded by the non-functional presumed *Mla* allele in cultivar Morex (*RGH1bcd*; WEI *et al.* 2002). All four cDNAs failed to confer disease resistance in the transient gene expression assay using A6 and K1 isolates. We tested two additional *B. g.* f sp *hordei* isolates on the four corresponding barley accessions and observed, as with the A6 and K1 isolates, full resistance except susceptibility on accession Nepal harbouring *Mla38-1* (not shown). None of the four identified cDNAs conferred disease resistance in the transient gene expression assay upon challenge with these two additionally tested *B. g.* f sp *hordei* isolates. The disease resistance response in *Mla16-1, Mla18-1*, and *Mla25*-containing plants must therefore be

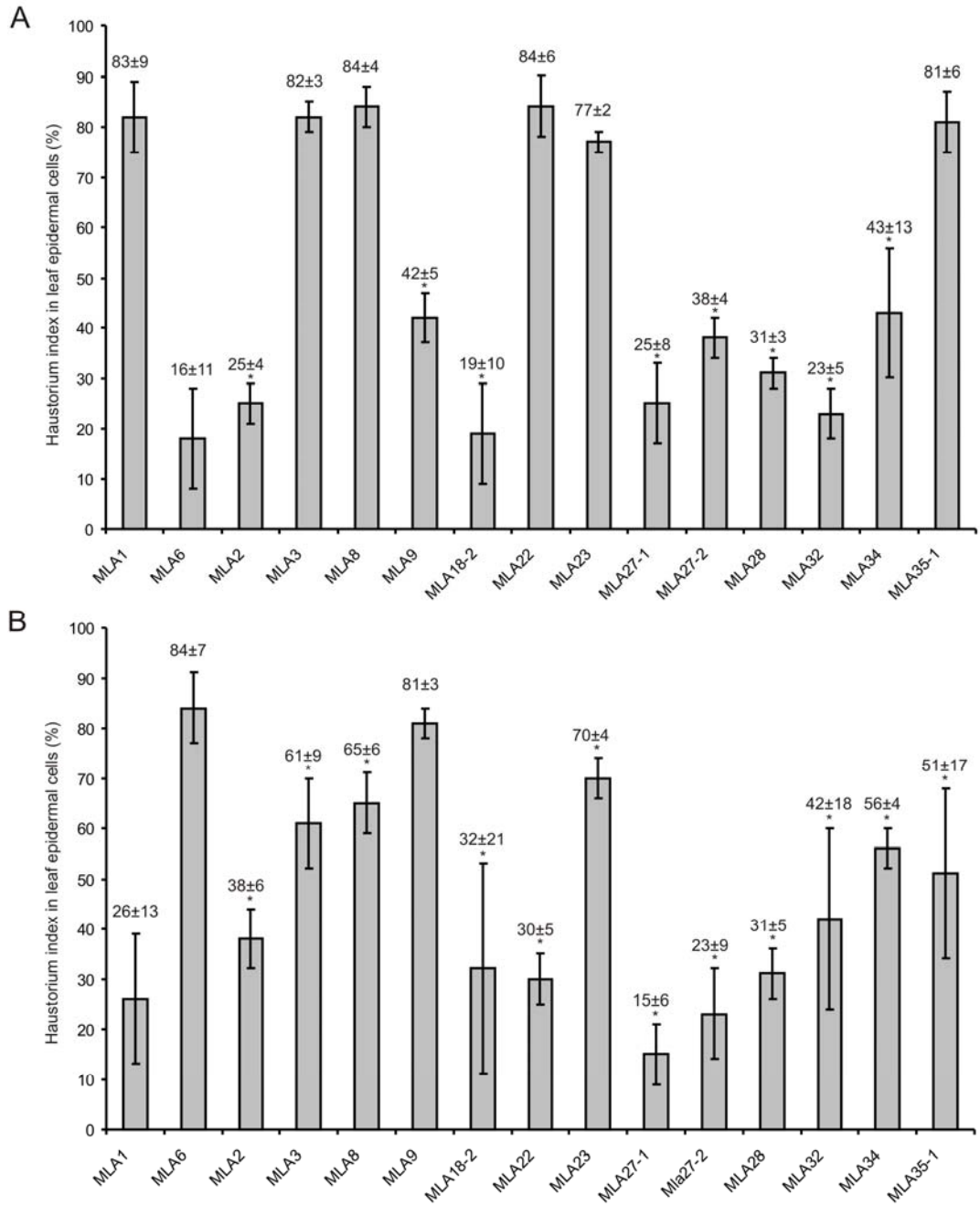


Figure 6: Functional analysis of candidate MLA cDNAs by transient single-cell gene expression. Plasmid DNA containing the indicated MLA cDNAs and a GUS reporter were co-delivered into epidermal cells of barley leaves. The leaves were incubated for 48 h after inoculation with powdery mildew conidiospores. GUS-stained single epidermal cells were then microscopically evaluated for the presence or absence of a fungal haustorium (haustorium index = % GUS-expressing epidermal cells attacked by a fungal sporeling that contain a haustorium). Data shown are based on the microscopic inspection of 50 to 100 interaction sites from at least four leaves and at least two independent biological replicates. MLA cDNAs labeled with an asterisk conferred a haustorium index that was different from the respective susceptible control (Student‘s t-test: $P < 0.05$). (A) Inoculation with *B. g.* f. sp. *hordei* isolate A6. MLA1 cDNA was used as a susceptible, MLA6 cDNA as a resistant control. (B) Inoculation with *B. g.* f. sp. *hordei* isolate K1. MLA6 cDNA was used as a susceptible, MLA1 cDNA as a resistant control. Note that MLA18-2, MLA27-1, MLA27-2, MLA28, MLA32 and MLA34 restrict fungal growth of both isolates. n.t. = not tested.

either conferred by a *R* gene other than *Mla* or these accessions contain other functional *Mla* homologues. Indeed, a second cDNA isolated from accession RS20-1 x Kibitz B, designated MLA18-2, conferred resistance to both A6 and K1 isolates in the transient gene expression assay (18% and 32% haustorium index, respectively; Figure 6) and this might explain the resistant infection phenotype seen on intact plants (IT *i*; Table 2). Taken together, it is possible that the divergent CC domains encoded by cDNAs MLA16-1, MLA18-1, and MLA25-1 contribute to the apparent non-functionality of the gene products.

Candidate cDNAs MLA3, MLA23, and MLA35-1 encode highly sequence-related proteins. MLA3 is six amino acids longer than MLA23 and differs by 13 residues in the C-terminus, whereas MLA35-1 differs from MLA23 by nine residues located in the LRR. Each of the candidate cDNAs conferred a similar haustorium index ranging from 50 to 70% upon inoculation with the K1 isolate, which is slightly but significantly lower compared to the control plasmid harbouring *Mla6* (84%; Figure 6). One possibility is that the weak K1 growth restriction is the result of a specific recognition of the corresponding AVR_A gene products. However, intact plants containing *Mla35-1*, *Mla3* or *Mla23* exhibited clearly disparate ITs ranging from full immunity (*i* in the *Mla35-1* containing line) to weak growth restriction (*ii* to *iii* in *Mla3* and *Mla23* containing lines, respectively) that were similar with both tested fungal isolates (Table 2). Thus, the observed weak K1 growth restriction upon single-cell over-expression of the highly sequence-related MLA3, MLA23, or MLA35-1 cDNAs could alternatively result from a weak 'non-specific' recognition of one or several inappropriate AVR_A effectors lacking in the A6 strain.

Plants of cultivar Golden Promise (*Mla8*) are fully susceptible to both A6 and K1 isolates (IT *v*; Table 2), indicating the absence of AVR_{A8} in both fungal strains. Unexpectedly, transient gene expression of the candidate MLA8 cDNA resulted in a moderate reduction of the haustorium index (65%) only in response to challenge with the K1 isolate (Figure 6). The protein encoded by this cDNA is identical with MLA1 up to residue 795 in the 10th LRR (see 6.1.). Expression of the MLA1 cDNA drastically reduces the haustorium index to 26% in a K1-specific manner (Figure 6). Thus, the differences between strong and weak resistance activities triggered by MLA1 and candidate MLA8 in the transient gene expression assay must be determined by the C-terminal 164 residues of the LRR. We conclude that, similar to the MLA3, MLA23, and MLA35-1 cDNAs, weak K1 growth restriction upon single-cell

over-expression of the candidate MLA8 cDNA might result from a weak 'non-specific' recognition of an inappropriate AVR_A effector that is absent in the A6 strain.

2.2.5. Identification of additional LRR motifs

The *Mla1* sequence was used to deduce a prototypical MLA protein of 958 amino acids containing within its N-terminal region (residue 1 to 160) the CC domain, a central NB-ARC domain (161 to 549), and C-terminal LRRs (550 to 958; ZHOU *et al.* 2001). The alignment of all 29 previously validated and candidate MLA sequences described in this study (Figure 4 and chapter 6.1.) enabled us to greatly improve the detection of structural motifs. Specifically, we detected the presence of four additional LRRs, which increases the total repeat number to 15 (HALTERMAN *et al.* 2003). These include the 1st LRR at position 557 (numbers refer to MLA1) and three additional imperfect LRRs at the C-terminal end (13th to 15th LRR; see 6.1.). The latter repeats including the 12th LRR are considered imperfect because of the lack of hydrophobic residues at the leucine positions in the LxxLxxLxLxx motif in some candidate MLA sequences. The 14th LRR has three variable positions instead of two after the second leucine of the LxxLxxLxLxx motif. The existence of these additional MLA LRRs was independently supported by the secondary structure prediction programs PSIPRED (BRYSON *et al.* 2005) and SCRATCH (CHENG *et al.* 2005), each predicting a short beta sheet for the respective LxxLxxLxLxx motifs. Of further note, the highly conserved acidic motif (G/D)A(H/Q)DDDL(C/M) at the very C-terminus of the deduced proteins appears to be a signature of MLA R proteins.

2.2.6. The LRR is highly diverse

25 validated and candidate MLA cDNA sequences were used to calculate the nucleotide diversity (π = 0.043) and nucleotide polymorphism (θ = 0.045) for the coding region (Figure 7A and Table 3). The MLA cDNAs encoding novel CC domains (MLA16-1, MLA18-1, MLA25-1 and MLA38-1) were omitted from this analysis to consider only the subset of MLA cDNA which share the same conserved CC domain. This nucleotide diversity is about 5-fold and 4-fold higher than the average values reported for cultivated (π= 0.0085) and wild barley (π = 0.0106) genomes, respectively (SAISHO and PURUGGANAN 2007). The region encoding the LRR was highly variable among all studied cDNAs, with nucleotide diversity increasing in a gradient towards the C-terminus (Figure 7A). After the 7th LRR, several sites were found that are occupied by five or six different amino acids. Additionally, the LRR domain had the

highest nucleotide diversity along the *Mla* gene with a π value of 0.074 (Table 3). A comparison of the sequence diversity of the 25 *Mla* sequences with 19 Arabidopsis *RPP13* alleles (entire gene: π = 0.045, θ = 0.040, (ROSE *et al.* 2004) showed that the nucleotide diversity of the LRR domain is almost identical for *RPP13* (π = 0.088).

Table 3: Nucleotide polymorphism and divergence of 25 candidate and validated MLA cDNAs

	entire cDNA	**CC domain**	**NB-ARC domain**	**LRR domain**
Number of sites	2850	453	1194	1203
Segregating sites:	478	27	131	320
θ	0.045	0.016	0.029	0.072
π	0.043	0.011	0.024	0.074
K [a]	0.145	0.198	0.109	0.161
Tajima's D [b]	-1.999	-1.196	-0.746	0.120
π_a / π_s	0.544	0.269	0.316	0.720
K_a / K_s [a]	0.467	0.672	0.279	0.584

[a] Divergence relative to the *Mla* homolog *HcMla*1 from H. *chilense*
[b] Total numbers of mutations were used for the calculation of Tajima's D value.

2.2.7. Positively selected sites are prevalent in the LRR domain

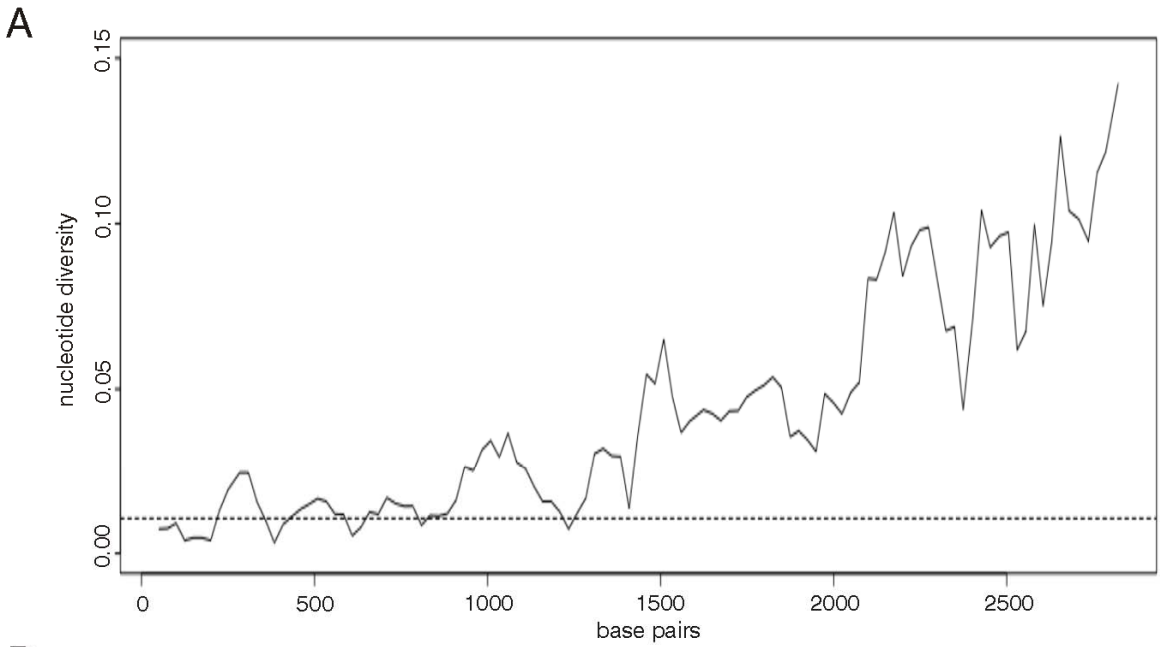
PAML version 4 (YANG 2007) was used to estimate nonsynonymous/synonymous ratios for the supposedly allelic sequences. We compared values of likelihood ratios for several nested models of codon substitutions (see chapter 2.4.). In all these analyses, the models that allow $K_a/K_s > 1$ were more accurate than the models where $K_a/K_s > 1$ is not permitted. This indicates that several nucleotide positions of the validated plus candidate *Mla* variants are under positive selection.

The analysis was initially performed for three sequence subsets. In one subset of 25 MLA cDNAs, we excluded the four sequences with unusually polymorphic CC regions (*Mla16-1, Mla18-1, Mla25-1 I* and *Mla38-1*). A second subset contained the 19 functionally validated *Mla* resistance specificities and a third calculation investigated both validated and candidate *Mla* sequences (28 variants). These three analyses gave similar results (Figure 7C, indicating that the choice of subsets has only a minor effect on the overall outcome. We conducted these analyses based on NJ trees and MP trees, and confirmed no significant differences indicating that these results are robust to the phylogenetic trees on which PAML analyses are based

(Table 4 and chapter 6.2.). Similar results were also obtained when we inspected a further sequence subset comprising only the nine candidate MLA cDNAs for which a resistance response to isolates A6 or K1 were undetectable (*Mla16-1, Mla18-1, Mla19-1, Mla25-1, Mla30-1, Mla31-1, Mla36-1, Mla37-1, Mla39-1*; data not shown).

When 25 validated and candidate MLA cDNA sequences were included, 34 positive selection sites with a posterior probability of p.p > 0.95 were identified (Figure 7B). Of these, one site was found in the CC region (at the C-terminal end near the hhGRExe motif of the NB-ARC domain (VAN OOIJEN *et al.* 2008), one site in the NB-ARC domain located in the RNBS-C motif and 32 in the LRR domain (Figure 7B and 7C). In the LRR, 18 residues were found to be under positive selection in the LxxLxLxx motif on the concave face of the LRR in the set of 25 *Mla* sequences (χ^2 test, $p < 1.36 \times 10^{-90}$; Figure 7B). It is postulated that the solvent-exposed concave site is critical for the recognition of a putative interaction partner (ELLIS *et al.* 2007).

Figure 7: Nucleotide diversity and positively selected sites in candidate and validated MLA cDNAs. A) Sliding window analysis of the nucleotide diversity (π) for 25 candidate and validated MLA cDNAs. MLA16-1, MLA18-1 and MLA25-1 were omitted from the analysis due to their divergent CC domains and MLA38-1 due to a stop codon at position 332. B) Amino acid residues are judged to be under positive selection if they are above a posterior probability of 0.95 (indicated by a dotted line). C) Illustration of the 15 LRRs of MLA1 that are conserved in all 25 MLA sequences. The first LRR starts at position 555. The LxxLxLxx sites, which are proposed to form a short, solvent-exposed β-strand motif (KAJAVA *et al.* 1995), are indicated. Note that the 14th LRR is irregular with three instead of two x positions after the first L position. Black: hydrophobic core residue, grey: site of any amino acid termed x in the LxxLxLxx motif. The 32 sites of positive selection are highlighted in orange. 18 of 32 positively selected sites in the LRR domain lie directly at an x position in the solvent-exposed LxxLxLxx motif. All shown positions of positive selection are statistically significant (posterior probabilities > 0.95) when looking at the set of 25 MLA sequences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0.00
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0.10
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0
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1000
1500
2000
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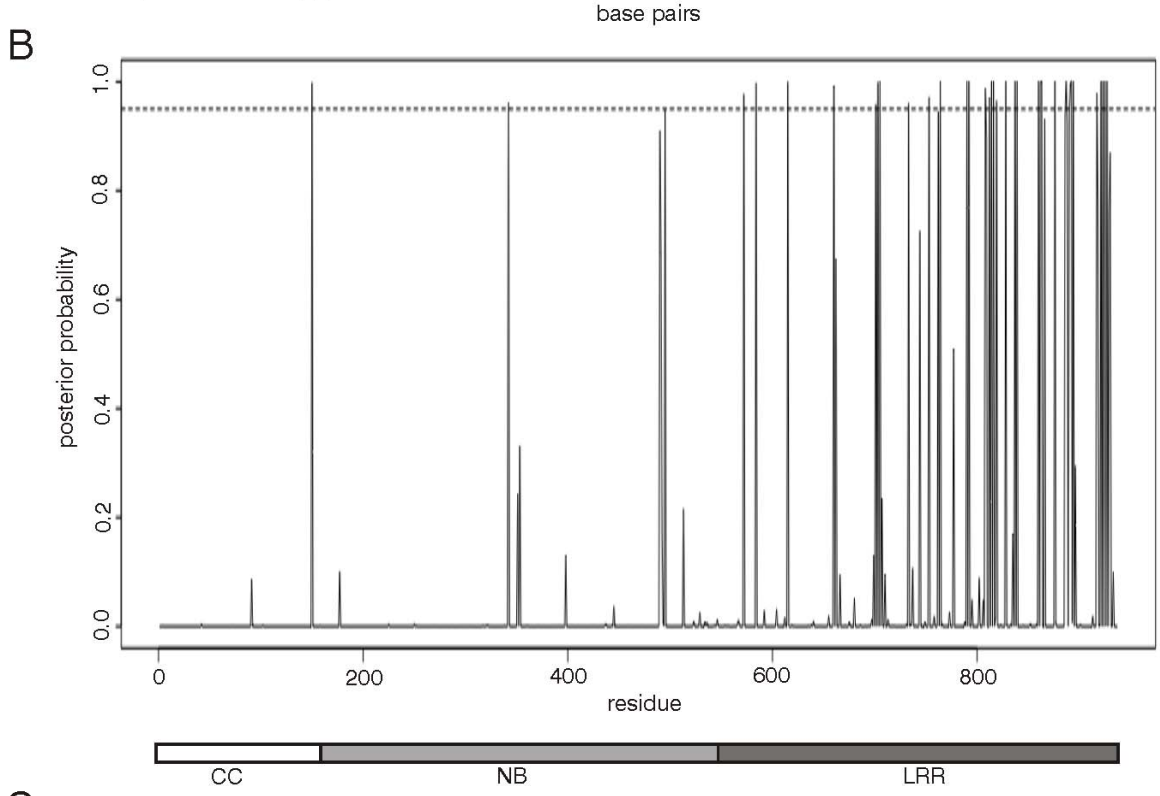
base pairs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0
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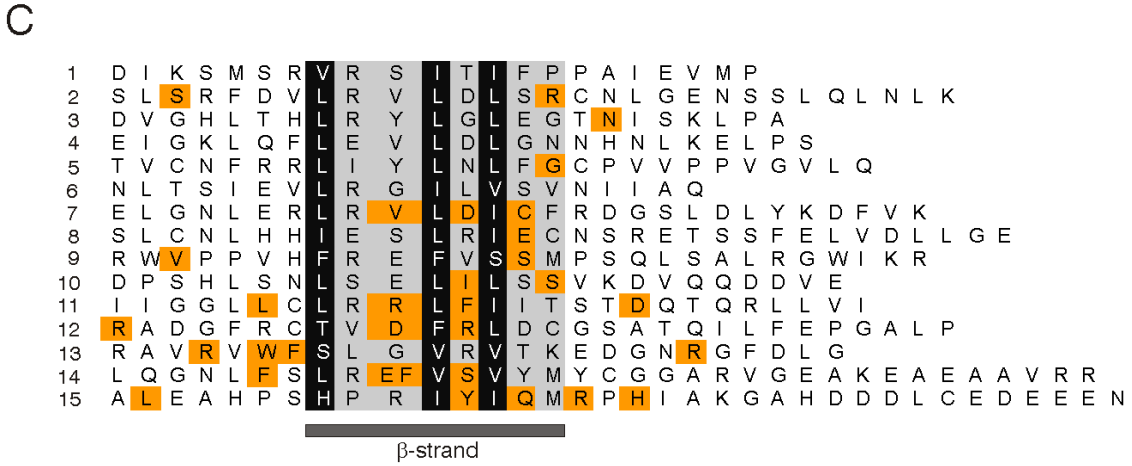
LRR


C
β-strand

The program Geneconv (SAWYER 1999) identified 96 significant (P<0.05) putative gene conversion or recombination tracks in 25 validated and candidate MLA cDNA sequences compared to each other (Figure 8). Gene conversion was detected mostly in the region containing the CC domain, the NB-ARC domain, and the first four LRRs until position 2100 (numbering refers to MLA1 cDNA). This suggests that the LRRs mostly experienced a different evolutionary history compared with the first part of *Mla*. We performed an analysis of the LRR starting at position 2100. The obtained data provided the same significant fit for models that allow $K_a/K_s > 1$ as obtained for the entire *Mla* gene and corroborate that the LRR is under diversifying selection (Table 4 and chapter 6.2.).

2.3 Discussion

The molecular analysis of *Mla* alleles present in a large set of barley cultivars has revealed 23 new candidate *Mla* variants in addition to the six previously known validated *Mla* alleles. 13 of these new candidate MLA cDNAs were shown to be functionally active. The LRR domain was found to be highly polymorphic with positively selected residues mostly found at the variable x position in the 15 LxxLxLxx motifs.

2.3.1. Isolation of candidate MLA cDNAs from barley lines with genetically defined *Mla* alleles

The powdery mildew pathogen has to be maintained on living plants, making long-term maintenance tedious. Therefore, most of the strains that were originally used to characterise the different *Mla* alleles described in literature are not available anymore. For this reason, it is impossible to determine whether our functional data from the transient assay are in agreement with the original characterization of race-specificities of the *Mla* alleles. In the case of the 13 functionally confirmed candidate MLA cDNAs, the resistance answers observed in the infection assay against isolates A6 and K1 (Table 2) correlate with the transient assay data (Figure 6).

There are two exceptions concerning the five functionally unconfirmed MLA cDNAs with CC domains like *Mla10* where the infection and transient assay gave different results: the barley cultivars harbouring *Mla19-1* and *Mla37-1* showed resistance against isolate K1 in the infection assay, but the cDNAs gave high haustorium indices in the transient overexpression.

In the case of these two candidate MLA cDNAs, it is unclear if the isolated sequence correspond to the specific *Mla* genes that were determined by allelism tests (JORGENSEN 1994). Therefore, either a second *Mla* variant that was not detected by our cloning strategy is responsible for this resistance, or a completely different powdery mildew resistance gene is present in the corresponding barley cultivars. For the nine MLA cDNAs with still undemonstrated resistance activity in the transient assay, including the two MLA cDNAs mentioned above and the three sequences with polymorphic CC domains, it remains to be seen if they confer resistance against other powdery mildew isolates than the two tested races K1 and A6. As all 23 new candidate *Mla* sequences were cloned from cDNA instead of from genomic DNA, they are transcriptionally active and are not derived from silent genes. Therefore, we assume that in most cases the newly cloned candidate MLA cDNAs correspond to the originally described *Mla* specificity of the tested barely cultivars.

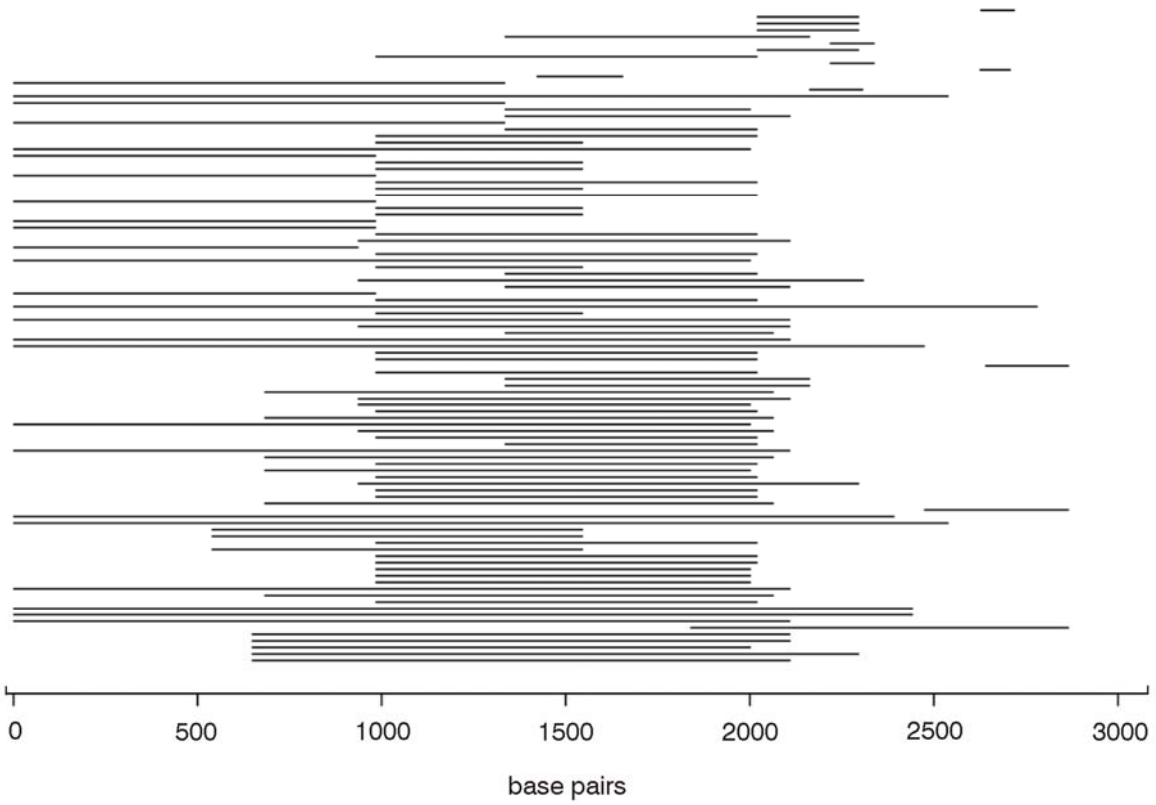


Figure 8: The program Geneconv (SAWYER 1999) was used to calculate gene conversion events in the 25 MLA cDNAs comparing per line each MLA sequence with only a second, different MLA sequence. We identified 96 putative gene conversion or recombination tracks ($P<0.05$). Global *P* values were assigned based on 10,000 permutations and corrected for multiple comparisons. Most gene conversion occurred in the CC and NB-ARC domain. There were only a few recombination events detected for the LRR domain from position 2100. The three domains of the *Mla* gene are indicated below.

As we have not detected any resistance response in the transient assay for the three cDNAs with polymorphic CC domains (*Mla16-1, Mla18-1* and *Mla25-1*), their function remains elusive. It will be interesting to test if they interact with the HvWRKY1/2 transcription factors as it was shown for the CC domain of MLA10 (SHEN *et al.* 2007). Possibly, these MLA varieties interact with other protein partners than MLA10.

Table 4: Likelihood ratio tests of positive selection for several MLA cDNA subsets

	M0 vs M3 (d.f. = 4)	**M1 vs M2 (d.f. = 2)**	**M7 vs M8 (d.f. = 2)**
28 MLA cDNAs	1522.23[a]	593.53[a]	652.62[a]
25 MLA cDNAs	1536.17[a]	598.40[a]	620.28[a]
19 MLA cDNAs	1070.10[a]	454.88[a]	488.19[a]
25 MLA cDNAs (MP tree)	1452.05[a]	562.57[a]	585.87[a]
25 MLA cDNAs (from position 2100)	619.79[a]	210.44[a]	233.48[a]

[a] P value < 0.01, d.f. = degree of freedom

2.3.2. Kinetics of the resistance response

It was shown previously that different *Mla* variants can trigger distinct resistance responses concerning the timing and the distribution of hypersensitive cell death (BOYD *et al.* 1995). *Mla3* and *Mla7* were shown to stop fungal growth at a later stage of the infection process than *Mla1* and *Mla6,* which terminated the infection early (BOYD *et al.* 1995). Our observation that *Mla3* and the two quite closely related sequences *Mla23* and *Mla35-1* exhibit an intermediate resistance in the transient expression assay are in agreement with the late response reaction observed in the isogenic Pallas line harbouring *Mla3* (Figure 6; BOYD *et al.* 1995).

Overexpression of the *Mla* alleles may accelerate a late response (SHEN *et al.* 2003). It was shown for *Mla12* that overexpression shifted the resistance response from post-haustorium growth arrest to an earlier abortion of fungal growth (SHEN *et al.* 2003). However, no differences were found in the resistance response when the fast reacting alleles *Mla1* and *Mla6* were tested under control of the native or the ubiquitin promoter (BOYD *et al.* 1995; SHEN *et al.* 2003). Thus, it seems that overexpression can affect the experimental outcome of the transient assay only in case of validated and candidate *Mla* variants that give a slow response. Since the transient assay by particle bombardment is an artificial system, the results

obtained do not necessarily reflect the absolute extent of the natural resistance response conferred by *Mla* variants, but rather indicate the relative strengths of the different specificities. For *Mla1* and *Mla6*, expression levels were shown to be similar and result in comparable protein levels (BIERI *et al.* 2004). Therefore, it seems feasible that MLA-containing recognition complexes depend on additional regulatory mechanisms that are responsible for the diverse MLA responses.

2.3.3. Organization of the *Mla* locus

The sequencing of the *Mla* locus in Morex showed that it is organized in a cluster of three homologous gene families (WEI *et al.* 2002). It was postulated that *RGH1bcd* corresponds to a truncated *Mla* allele in the cultivar Morex because this partial gene shared the highest sequence similarity with the already isolated alleles *Mla1, Mla6* and *Mla13* (WEI *et al.* 2002). Comparative sequence analysis of the 29 validated and candidate MLA cDNAs showed that all of them had the highest sequence similarity with *RGH1bcd* in comparison to the other three *RGH1* genes. Two genetics studies that examined the fine structure of the *Mla* locus could not completely clarify whether *Mla* variants are allelic or closely linked genes (GIESE *et al.* 1981; WISE and ELLINGBOE 1985). Our Southern blot analysis showed that the locus organization seems to vary strongly among barley cultivars (Figure 5).

The complexity of the *Mla* locus is further indicated by the presence of two expressed genes with different sequences in a single barley cultivar in two cases. In barley cultivar RS1-8 x Piccolo E, *Mla27-1* and *Mla27-2* share a sequence identity of 99%. In the second cultivar RS20-1 x Kiebitz B, *Mla18-1* and *Mla18-2* have completely different sequences (Figure 4). These two cases suggest the presence of two different *Mla* genes instead of alleles, as these lines were homozygous after several selfings. It is postulated that unequal crossing over or tandem duplication are important mechanisms to create new NB-LRR genes (MCDOWELL and SIMON 2008). As the three homologous *Mla* gene families are organized as a cluster (WEI *et al.* 2002), we suggest that gene duplication or a similar recombination event occurred in the two cases described above. Still, we assume that most of the isolated sequences are true alleles of *RGH1bcd*. It will be essential to sequence the complete *Mla* locus of several resistant barely cultivars to shed light on the mechanisms that lead to the high polymorphism at the *Mla* locus, and to clarify definitely the question of allelism.

2.3.4. Diversity of the LRR domain

Why are validated and candidate MLA cDNAs so remarkably polymorphic in the LRR domain? Domain swap experiments between MLA1 and MLA6 indicate that the recognition specificity is determined exclusively by the LRR domain (SHEN *et al.* 2003). Still, it remains to be investigated if there is a direct or indirect recognition of the cognate fungal effectors by MLA proteins. The two postulated powdery mildew effector genes AVR_{A10} and AVR_{K1} are members of a large gene family (RIDOUT *et al.* 2006). Thus, it is possible that the corresponding powdery mildew effector proteins show high levels of polymorphism and are detected by a diverse array of MLA proteins. Direct interaction is proposed to be a driving force for the coevolution of new effector molecules to avoid detection and R proteins with new recognition abilities (MCDOWELL and SIMON 2006). Thus, the availability of a large set of validated and candidate MLA cDNAs together with their cognate still to-be-cloned *AVRMla* effectors, will be an excellent source to study function and evolution of this interaction. Interestingly, the diversity observed for *Mla* is comparable to the *Arabidopsis RPP13* alleles (ROSE *et al.* 2004). Both the *RPP13* gene and the corresponding effector gene *ATR13* are extremely polymorphic and both were shown to be under diversifying selection (ALLEN *et al.* 2004).

2.3.5. Positive selection was found for the *Mla* genes

With 32 out of 34, the majority of positively selected sites were mapped in the LRR domain of MLA (Figure 7). Similar observations were made for the flax *L* locus, where the encoded N-terminus is probably involved in recognition specificity and shows evidence for diversifying selection at some positions (ELLIS *et al.* 1999; LUCK *et al.* 2000). Additionally, a study of positively selected sites in *Arabidposis* NB-LRR genes also found some sites lying either in the CC or TIR NB-ARC region (MONDRAGON-PALOMINO *et al.* 2002). It was suggested that these residues play a role in intramolecular folding and have coevolved with the LRRs to optimize interaction specificities (MCDOWELL and SIMON 2006; MONDRAGON-PALOMINO *et al.* 2002). The NB-ARC domain of R proteins has been proposed to function as a molecular switch, where the NB-LRR protein is in an inactive state and the CC and the LRR domains interact with the NB-ARC region intramolecularly (TAKKEN *et al.* 2006). After interaction with a pathogen effector and induction of the resistance response, the NB-LRR protein would then undergo a conformational change (TAKKEN *et al.* 2006).

In MLA, 18 of 34 positively selected residues lie in the variable x positions of the LxxLxLxx motifs on the solvent-exposed surface (Figure 7C). This finding correlates with studies on flax *L*, *RPP13* and recently *Pi-ta,* where positive selection was found to occur mostly in the solvent-exposed residues of the LRR domain (ELLIS *et al.* 1999; HUANG *et al.* 2008; ROSE *et al.* 2004). It was proposed that solvent-exposed residues of the LRR domain are involved in the interaction with another protein (DUNNING *et al.* 2007), and it is possible that the positions that are under positive selection interact (directly or indirectly) with the cognate pathogenic proteins to create new possibilities of recognition. However, it remains to be investigated if polymorphisms in the *R* gene and the cognate effector gene are an indication for direct protein-protein interaction (ELLIS *et al.* 2007).

So far, only four examples of different NB-LRR *R* genes were investigated that showed direct interaction with the effector proteins(DESLANDES *et al.* 2003; DODDS *et al.* 2006; JIA *et al.* 2000; UEDA *et al.* 2006). The *Mla-AvrMla* system could be an additional interesting model to explore if diversifying selection correlates with direct interaction. The isolation of more *AvrMla* effector genes is essential to further characterise the 29 validated and candidated MLA cDNAs. A suitable assay will have to be established to test each *Mla* sequence against each isolated fungal effector to group them according to recognition specificity. The obtained information will help to answer the questions why *Mla* is so highly polymorphic and if the positively selected sites in the LRR domain are involved in a direct or indirect recognition.

2.4 Material and methods

2.4.1. Plant material and barley powdery mildew isolates

The plant material used for this study was derived from seeds obtained from the stock centers at the IPK Gatersleben (Germany), NGB (Sweden), USDA National Small Grains Research Facility (USA), and INRA-Clermont (France). New candidate MLA cDNAs were isolated from barley cultivars described in Table 2. No *Mla* amplicons could be obtained from the following lines: RS 170-47 x Kiebitz B (*Mla17*), Diamant x 1B-20 (*Mla26*) and 110-4 x Sonja (*Mla29*). *Mla7* was found in Gopal (suggested to harbour *Mla5*, PI 41162). *Mla8* was found in IGV3-011 described to have *Mla11*, and *Mla*25 was isolated from RS145-39 x Kiebitz B (proposed to possess *Mla20*). The following barley cultivars were also used for the Southern blot analysis: Engledow India (Clho 7555), Galleon (CFH 4877), Ingrid (PI 263574), Manchuria (PI 69642), Morex (Clho 15773) and Ingrid isogenic (No. 22, *Mlk1*) (IGV3-004).

The *Blumeria graminis* f. sp. *hordei* strains A6 (*AvrMla3, AvrMla6, AvrMla7, AvrMla9, AvrMla10, AvrMla12,* and *AvrMla13*) and K1 (*AvrMla1, AvrMla3, AvrMla7, AvrMla13,* and *AvrMla22*) were maintained on barley cultivar Pallas P01 or on Pallas P03, respectively. The strains DH14, CC52 maintained on barley cultivar Golden Promise were used additionally for functional analysis and for infection assays. For all experiments, plant material or detached leaves with or without fungal spores were kept at 20°C, 70% relative humidity and 16 h light/8 h dark cycle.

2.4.2. Isolation and cloning of candidate MLA cDNAs

RNA was extracted from seven day old barley seedlings with TRIZOL® Reagent (Invitrogen) and cDNA was produced using 5 µg of total RNA in a final reaction volume of 10 µl by the Superscript III reverse transcriptase (Invitrogen), following the manufacturer's protocols. 3'-RACE, 3'-UTR PCR and cDNA PCR amplifications were carried out using the Expand High Fidelity^{PLUS} PCR System (Roche) following the manufacturer's manual. To amplify the 3 kb long cDNA fragment, 5% DMSO was included in the 50 µl PCR reaction mix and 2 µl of 5x diluted cDNA was used as a template. All PCR reactions were performed in a PTC-200 thermocycler (Bio-Rad). The amplification conditions to obtain 3'-RACE products using the primers sbi178/sbi480 (all primers are listed in chapter 6.6.) were 2 min at 94°C, followed by 35 cycles of 20 sec at 94°C, 30 sec at 50°C, 90 sec at 72°C, and then a final extension for 7 min at 72°C. 3'-UTR PCR was carried out as an alternative strategy to 3'-RACE using primers sse011/sse012 with the same PCR conditions as for 3'-RACE. The approximately 600bp long 3'-RACE or 3'-UTR PCR amplification products were purified by the GenElute^{IM} Gel Extraction Kit (Sigma-Aldrich) and cloned using the pGEM®-T easy vector systems (Promega) following the manufacturer's protocols. To amplify the 23 different candidate MLA variants using cDNA as a template, the different primers were used listed in chapter 6.6. The PCR conditions were 2 min at 94°C, followed by 10 cycles of 20 sec at 94°C, 30 sec at 50°C, 4 min at 68°C, followed by 20 cycles of 20 sec at 94°C, 30 sec at 55°C, the elongation time was 4 min plus additional 10 sec for every additional cycle at 68°C, and then a final extension for 7 min at 68°C. The 3 kb long PCR amplification products were purified using the GenElute^{IM} Gel Extraction Kit and were cloned in a modified pENTR4 (Invitrogen) containing an ubiquitin promoter. Amplification products and the vector were digested with the restriction enzymes *Asc*I and *Not*I (New England Biolabs) and ligated. All isolated candidate MLA cDNAs were verified by a second independent amplification step from newly

generated cDNA. Thus, a 25 µl PCR mix was obtained using the Expand High Fidelity^{PLUS} PCR System under the same conditions mentioned above and after 2 min at 94°C, 6 cycles of 20sec at 94°C, 30 sec at 50°C, 4 min at 68°C, followed by 7 min at 68°C, the generated product was used as a template for the *Pfu*Ultra high-fidelity DNA polymerase (Stratagene). These specific PCR reaction contained 1 µl of the generated template, 5 µl buffer, 3 mM $MgCl_2$, 200 µM each dNTP, 0.4 µM each primer, 10% glycerol and 2.5 units of polymerase in a final volume of 50 µl. The amplification conditions were 2 min at 94°C, followed by 32 cycles of 20 sec at 94°C, 30 sec at 50°C, 10 min at 68°C, and then a final extension for 7 min at 68°C. All PCR-generated clones were sequenced using Big Dye Terminator V3.1 following the manufacturer's protocol on an ABI3730 automated sequencer (Applied Biosystems). To verify each isolated MLA cDNA, six clones from two independent amplification events were sequenced. Because a PCR primer derived from the coding region of *HcMla1* was used to amplify *Mla18-1*, the 5'-end directly after the start codon was confirmed as follows: 5'-RACE was carried out with the SMART^{TM} RACE cDNA Amplification Kit (Clontech) following the manufacturer's instructions. The PrimeScript reverse transcriptase (Takara) was used to generate cDNA from isolated RNA of cultivar RS20-1 x Kiebitz. The gene specific primer sse078 was designed for 5'-RACE. The touchdown conditions for 5'-RACE were 5 cycles of 30 sec at 94°C, 3 min at 72°C, 5 cycles of 30 sec at 94°C, 30 sec at 70°C and 3 min at 72°C, after 2 min at 94°C followed by 25 cycles of 30 sec at 94°C, 30 sec at 68°C and 3 min at 72°C were performed followed by 7 min at 72°C.

2.4.3. Single-cell transient expression assay

The single-cell transient expression assay was carried out as described in (SCHWEIZER *et al.* 1999; ZHOU *et al.* 2001) with some modifications. After seed vernalization at 4°C for 24h, barley cultivar Ingrid *mlo5* was grown for six days in a growth chamber. The reporter plasmid pV26-UMUG (BIERI *et al.* 2004) encoded the β-glucuronidase (GUS) gene and the *Mlo* gene controlled by the ubiquitin promoter. The gold particles (1.0 µm diameter, Bio-Rad) were coated with totally 2µg of a mixture of reporter plasmid and pENTR containing *Mla* controlled by a ubiquitin promoter in a molar ratio of 1:1. Single leaves were transformed with a particle inflow gun of the model PDS-1000/He (Bio-Rad) and after 4h, inoculated with spores of an appropriate powdery mildew isolate at high inoculation density. After an incubation time of 48h, the samples were vacuum-infiltrated with GUS staining solution and incubated at 37°C overnight (ZHOU *et al.* 2001). Finally, the samples were destained with two

volumes of ethanol and one volume of 50% glycerol, 25% lactic acid and 25% water. The interaction between GUS-expressing cells and powdery mildew spores was observed by light microscopy (SHEN *et al.* 2003). An appressorium that was blocked by an epidermal GUS-expressing cell was counted as an incompatible interaction, whereas a developed haustorium was counted as a compatible interaction. The haustorium index is the ratio of the number of compatible interactions to the number of total interactions. Per experiment, 100 interactions were counted with at least one independent repetition.

2.4.4. Southern blot

Isolation of genomic DNA from 33 barley cultivars and Southern hybridization were performed as described by STEIN *et al.* (2000). The genomic DNA was digested with *Eco*RI (New England Biolabs) before hybridizing with the probe. The primers sbi398/sbi327 (see chapter 6.6) were used to amplify a 501bp long probe from the region encoding the second half of the NB-ARC domain of *Mla*1 derived from the template cosmid p6-49-2 (ZHOU *et al.* 2001) with *Taq* DNA Polymerase (Sigma) following the manufacturer's instructions.

2.4.5. Data analysis

PAML version 4 (YANG 2007) was used for the estimation of nonsynonymous-synonymous rate ratio for the *Mla* sequences (performed by Takashi Tsuchimatsu). The likelihoods of more complex models was compared to null models M0 (which assumes neutral codon evolution) and M1a (nearly neutral codon evolution with two codon classes allowed to take on values from $0 \leq \omega_0 \leq 1$ or $\omega_0 = 1$) with those of a more complex model M2a, which incorporates an additional positively selected sites class ($\omega > 1$), and M3 (which assumes three site classes). We also examined the results of analyses using model M7 and M8, which both assume a β-distribution for $0 \leq \omega \leq 1$, with the latter model allowing for an extra class of sites with ($\omega > 1$). We tested for positive selection by comparing three times the log-likelihood differences of M0 vs M3, M1a vs M2a, and M7 and M8. The codeml package of PAML version 4 was used to calculate posterior probabilities of codon sites that are under positive selection. We calculated posterior probabilities (p.p.) that each codon sites belongs to one of the selection classes. Codons with a significant posterior probability (p.p.>0.95) of being in a positively selected class ($\omega > 1$) are considered likely to have experienced postive selection detected all in M2a (Bayes empirical Bayes method), M3, and M8 (Bayes empirical Bayes

method). Amino acid position 825 and 826 were removed from all these positive selection analyses because of alignment ambiguity. Phylogenetic trees generated for PAML analysis were calculated using MEGA 4 (TAMURA *et al.* 2007) for Neighbor-joining (NJ) phylogenetic trees and Phylip 3.67 (FELSENSTEIN 1989) for Kimura 2-parameter models and for maximum parsimonious (MP) trees (Table 4 and chapter 6.2.). Population genetic analysis were performed with DnaSP version 4.10.7 (ROZAS *et al.* 2003) and gene conversion was detected with GENECONV version 1.81a (SAWYER 1999) described by MONDRAGON-PALOMINO and GAUT (2005). Global *P* values were assigned based on 10,000 permutations and corrected for multiple comparisons. DNA sequence data was analyzed with Clone Manager Profession Suite version 8 and alignments were modified in GeneDoc version 2.7 (NICHOLAS *et al.* 1997). Coiled-coils were analyzed with COILS version 2.2 (LUPAS *et al.* 1991). The EMBOSS package (RICE *et al.* 2000) was used to search for LRR motifs. They were confirmed by secondary structure prediction using the PSIPRED and SCRATCH server (BRYSON *et al.* 2005; CHENG *et al.* 2005).

3. Function of different MLA coiled-coil domains

3.1 Introduction

The transcription factors of the WRKY family are involved in various processes such as pathogen defence, seed development and senescence (MANGELSEN *et al.* 2008). All members of this transcription factor family consist of a DNA-binding WRKY domain, which contains the highly conserved WRKY-GQK motif and a zinc finger motif. HvWRKY1 and HvWRKY2 were shown to be repressors of MAMP-triggered immunity in barley. They were demonstrated to interact with the first 46 residues of the MLA10 coiled-coil (CC) domain (SHEN *et al.* 2007). It is postulated that upon effector-triggered activation of MLA10, the coiled-coil domain can directly interact with the HvWRKY1/2 proteins and thereby interfere with their repressor function to activate defence genes (SHEN *et al.* 2007). So far, 28 different candidate and validated MLA cDNAs have been isolated. In 25 MLA sequences, only three amino acid polymorphisms were found in the first 46 residues compared to the sequence encoded by *Mla10* (Chapter 2). In contrast, the three candidate MLA cDNAs *Mla16-1*, *Mla18-1* and *Mla25-1* are more diverse in the encoded CC domain (Chapter 2).

Besides the study about WRKY interaction, several other CC-NB-LRR proteins have been described that form complexes through their CC domain with their cognate interaction partners: *Arabidopsis* RPM1 with RIN4, *Arabidopsis* RPS5 with BPS1, tomato Prf with Pto, and potato Rx with RanGAP2 (RAIRDAN *et al.* 2008). Thus, it seems that the CC domain is involved in signalling and effector recognition. Recently, a study revealed the highly conserved EDVID motif, which was demonstrated to be involved in intramolecular folding of the R protein Rx (RAIRDAN *et al.* 2008). The EDVID motif is located at the beginning of the CC domain and was shown to be a highly conserved motif in all CC-NB-LRR proteins (RAIRDAN *et al.* 2008). All candidate and validated MLA cDNAs including *Mla18-1*, *Mla25-1* and the wheat *Mla* homologue *TmMla1* (Tina Jordan, unpublished data) contain the EDVID motif with a valine instead of the isoleucine at position 80 (see chapter 2).

To further investigate the role of the CC domain in MLA, we have performed a domain swap analysis between different N-termini of MLA18-1, MLA25-1 and domains of MLA1. Furthermore, it was tested if MLA18-1 and MLA25-1 physically interact through their N-termini with the two transcription factors HvWRKY1 and HvWRKY2. No resistance function was detected in chapter 2 for the two *Mla* variants *Mla18-1* and *Mla25-1*. Thus, we wanted to

examine if mutations in the highly conserved MHD motif of the ARC2 domain result in a spontaneously induced HR defined as autoactivation. These findings would indicate that the proteins are functional.

3.2 Results

3.2.1. Diversity in the encoded N-terminus by three *Mla* genes and comparison to a wheat homologue

For the resistance gene *Mla*, 28 different sequences were isolated as described in chapter 2. 25 MLA proteins were nearly identical in the first 46 residues, with only three amino acids polymorphisms. In contrast, MLA16-1, MLA18-1 and MLA25-1 were more polymorphic in the first 46 amino acids compared to MLA10. MLA18-1 and MLA16-1 differ at 16 positions from MLA10, and MLA25-1 differs from MLA10 at 12 amino acids. Furthermore, MLA18-1 and MLA16-1 are identical in the CC-domain and differ only at 11 amino acids from each other. These polymorphisms are distributed over the complete length of the sequence. The N-terminus of MLA25-1 is slightly different to MLA18-1 with six amino acid differences. The first 46 residues encoded by the *Mla* homologue *TmMla1* isolated from *Triticum monococcum* (Tina Jordan, unpublished data) was also included in this study. The region of *TmMla1* encoding the first 46 residues is more closely related to the 5'end of *Mla10* with a sequence identity of 88% than to *Mla25-1* with a sequence identity of 81% or to *Mla18-1* with 76%.

No resistance was conferred by the three candidate MLA cDNAs MLA16-1, MLA18-1 and MLA25-1 when these sequences were transiently overexpressed in barley and tested against four powdery mildew isolates (Figure 9 and chapter 2). *TmMla1* neither showed any resistance activity against the four powdery mildew isolates when overexpressed in barley. We wanted to further investigate if *Mla16-1, Mla18-1*, *Mla25-1* and the wheat homologue *TmMla1* might be involved in resistance specificity against powdery mildew. One possibility would be to perform a screen for resistance activity to find an appropriate powdery mildew isolate. As such an approach would be quite laborious for all four *Mla* variants, we performed domain swap analysis as described between *Mla1* and Mla6 (SHEN *et al.* 2003) to investigate their functional activity.

3.2.2. Design and functional characterisation of domain swap constructs

Because MLA10 interacts through its first 46 residues of the CC domain with the transcription factors *Hv*WRKY1 and *Hv*WRKY2 (SHEN *et al.* 2007), we wanted to examine the function of the different MLA coiled-coil domains. Thus, chimeras were generated (Simon Schwizer, Master thesis) where the first 46 residues of MLA1 were replaced with MLA18-1 or MLA25-1, called Swap2 or Swap5, respectively (Figure 10A). Two additional domain swaps of MLA18-1 and MLA25-1, where the LRR domain was replaced with MLA1, were called Swap3 and Swap6, respectively (Figure 10A, Simon Schwizer, Master thesis). These domain swap constructs were designed in a way analogous to the chimera called 61111 of MLA6, where the LRR domain (550-956) was replaced with the corresponding MLA1 sequence. The MLA1-specific resistance against powdery mildew isolate K1 was retained for this domain swap construct (SHEN *et al.* 2003).

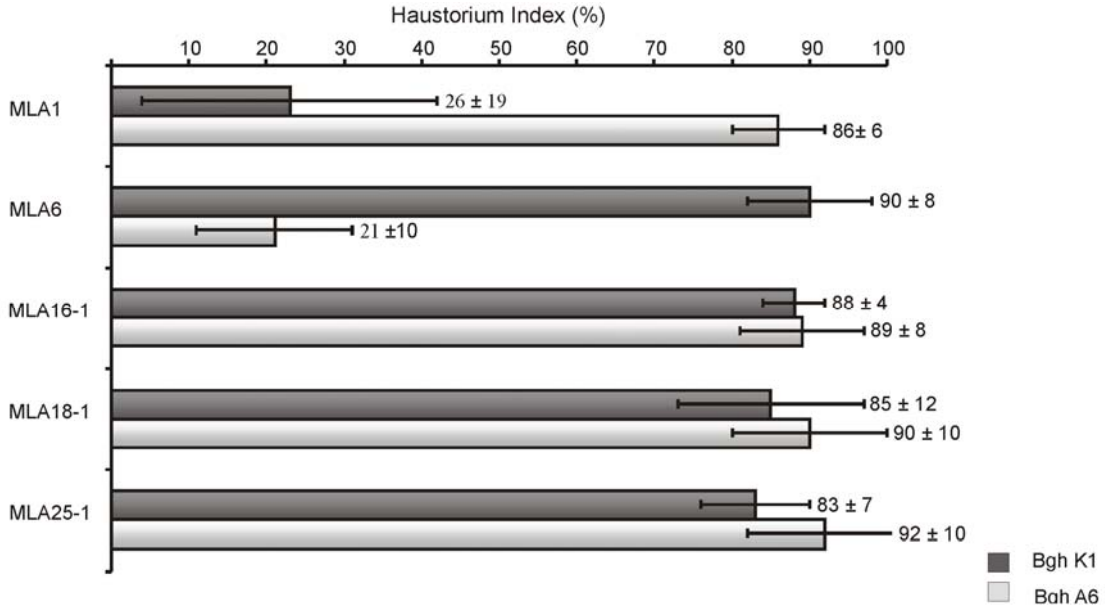
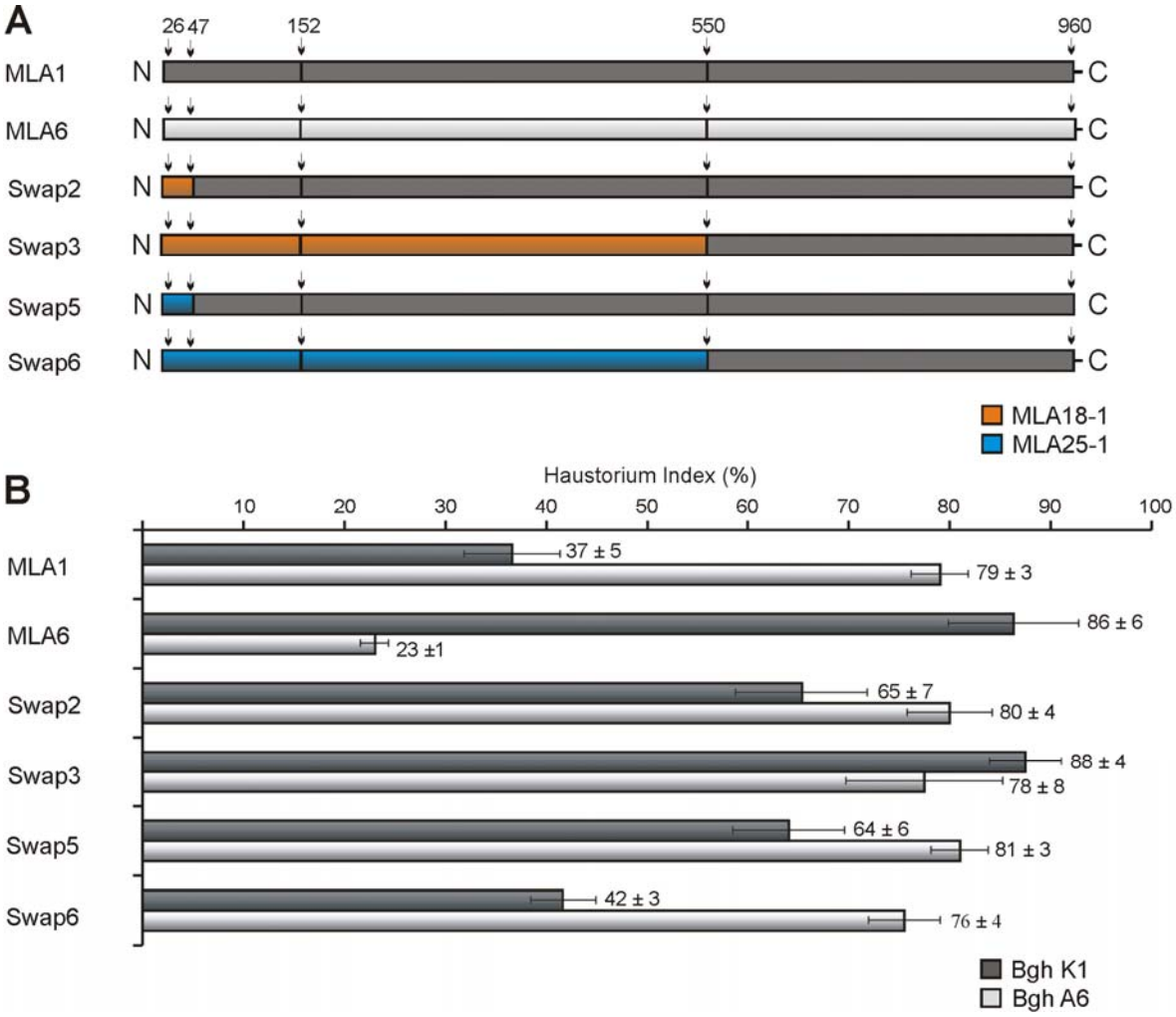


Figure 9: Transient overexpression of MLA cDNAs together with the reporter gene β-glucuronidase (GUS) in barley leaves and incubated for 48 h with spores of powdery mildew isolates Bgh K1 or Bgh A6. The experiments were performed once or twice per construct. MLA1 confers resistance against Bgh K1, but susceptibility against Bgh A6, whereas MLA6 was used as a negative control for Bgh K1 and as a positive control for Bgh A6. MLA16-1, MLA18-1 and MLA25-1 resulted in susceptible interactions against both isolates K1 and A6.

All four domain swaps were transiently overexpressed in barley leaves and tested against the two fungal isolates A6 and K1 for resistance response (Simon Schwizer, Master Thesis). It was found that the two candidate cDNAs MLA18-1 and MLA25-1 conferred no resistance activity when tested against isolates A6 and K1 (Chapter 2, Figure 9). As a positive control, MLA1 was resistant against isolate K1, and MLA6 as negative control, was susceptible (Figure 10B). All four chimeras were susceptible when tested against the isolate A6, giving the same result as the unchanged MLA1 cDNA. The expression of the two chimeras Swap2 and Swap5 resulted in haustorium indices of 65% and 64% against isolate K1 (Figure 10B). Thus, the exchange of the first 46 residues resulted in a weak resistance response against K1, which is significantly different ($P \leq 0.05$) from the haustorium index of 37% for MLA1 and from the susceptible control MLA6 with a haustorium index of 86% (Figure 10B). Construct Swap6 gave a high resistance response with a haustorium index of 42%, which is comparable with the response triggered by MLA1. The resistance response against isolate K1 showed by Swap6 corresponds to previously obtained results by expressing a similar chimera of the CC-NB-ARC domain encoded by the MLA6 cDNA and the LRR encoded by the MLA1 cDNA. This domain called 61111 was resistant against K1 and susceptible against A6 as shown for MLA1 (SHEN *et al.* 2003). In contrast, Swap3 was fully susceptible in the transient assay with a haustorium index of 88% against K1. To examine if Swap3 was expressed on the protein level, all four swaps were cloned as C-terminal HA epitope-tagged constructs. All of them were expressed during the transient assay determined qualitatively by immunoblotting (data not shown, Simon Schwizer, Master thesis).

Figure 10: A) Schematic illustration of the four different chimeric proteins between MLA18-1/MLA25-1 and MLA1. MLA1 is indicated in dark grey and MLA6 in light grey. Swap2 is a chimera between the first 46 residues of MLA18-1(orange) and MLA1 (grey) for the rest of the protein. Swap3 consists of the LRR domain$_{550\text{-}960}$ of MLA1 (grey) and the CC-NB-ARC of MLA18-1 (orange). Swap5 is a construct between the N-terminus$_{1\text{-}46}$ of MLA25-1 (blue) and MLA1 (grey) for the rest of the protein. Swap6 is a chimera of MLA25-1 (blue) with the LRR domain$_{550\text{-}960}$ of MLA1 (grey). B) Different *Mla* constructs were transiently overexpressed together with the reporter gene β-glucuronidase (GUS) in barley leaves and incubated for 48 h with spores of powdery mildew isolates Bgh K1 or Bgh A6. GUS-stained epidermal cells were microscopically evaluated for compatible or incompatible interactions in at least two independent experiments. The haustorium index is the fraction of incompatible interactions in percent. MLA1 confers resistance against Bgh K1 and susceptibility against Bgh A6. MLA6 was used as a negative control for Bgh K1 and as a positive control for Bgh A6. The haustorium indices of the four Swap2, Swap3, Swap5 and Swap6 illustrated and explained in A) are indicated in B).



3.2.3. Functional characterization of the coiled-coil domains

We wanted to examine if the first 46 residues of MLA18-1, MLA25-1 and TmMLA1 physically interact with *Hv*WRKY1 and *Hv*WRKY2 in the yeast two-hybrid assay. Thus, bait constructs of all four N-termini fused to the LexA binding domain were designed as described for $MLA10_{1\text{-}46}$ (Figure 11A; SHEN *et al.* 2007). It is known that only the C-terminal domain of HvWRKY1 or HvWRKY2 is essential for physical interaction with $MLA10_{1\text{-}46}$ (Armin Töller and Paul Schulze-Lefert, MPIZ Köln, personal communication). Thus, the two C-termini, *Hv*$WRKY1_{260\text{-}353}$ and *Hv*$WRKY2_{243\text{-}319}$, were used as prey constructs fused to the B42 activation domain (Figure 11B). As a negative control, a prey with the WRKY domain (178-242) was used. The WRKY domain is the DNA-binding domain of the transcription factor *Hv*WRKY2. Protein-protein interactions were analysed *in vivo* on agar plates, where the substrate X-gal is converted to an insoluble blue indigo dye in the presence of β-galactosidase (see chapter 3.4.4.). All four different N-termini of MLA10, MLA18-1, MLA25-1 and TmMLA1 were tested against $HvWRKY1_{260\text{-}353}$ and $HvWKRY2_{243\text{-}319}$ (Figure 11C-D). As a

positive control, the physical interaction between MLA_{1-46} and the C-termini of HvWRKY1/2 was examined. After an incubation time of 48 h, the colonies of the positive control turned blue. After 72h, the interaction between $TmMLA1_{1-46}$ and $HvWRKY1_{260-353}$ resulted into a weak blue colour, while the positive control already turned dark blue (Figure 11C). The N-terminus neither of MLA18-1 nor of MLA25-1 interacted with $HvWRKY1_{260-353}$ and $HvWKRY2_{243-319}$. Thus, the differences in the first 46 residues are at positions important for the contact interface, of MLA and HvWRKY1/2.

3.2.4. Autoactivating histidine-to-alanine mutations in the VHD motif of MLA

The highly conserved MHD motif lies in the ARC2 domain that is probably important for protein activation (see 1.2.2.). In the crystal structure of human Apaf-1 with bound ADP (contains a homologous NB-ARC domain), the histidine residue of the corresponding MHD motif LHD provides a hydrogen bond to the β-phosphate of ADP (RIEDL *et al.* 2005). Thus, it is likely that this motif is associated with the ATP binding pocket and detects the nucleotide binding status (RAIRDAN and MOFFETT 2007). NB-LRR proteins with mutations in the MHD motif can result in spontaneously induced HR. Such autoactivated proteins were generated by introducing an aspartate to valine mutation into potato Rx, flax L6, tomato I-2 and Mi-1, and into tomato NRC1, a protein that is required by many R proteins to initiate HR signalling (BENDAHMANE *et al.* 2002; GABRIELS *et al.* 2007; HOWLES *et al.* 2005; VAN BENTEM *et al.* 2005; VAN OOIJEN *et al.* 2008). The histidine is the only invariable residue of the MHD motif through all NB-LRR proteins recently investigated by VAN OOIJEN *et al* (2008). Thus, the histidine to alanine mutation was shown to be the most effective single mutation resulting in autoactivation of the R proteins Mi-1 and I-2 (VAN OOIJEN *et al.* 2008).

In MLA, the conserved MHD motif has a valine instead of a methionine, resulting in a VHD motif. We wanted to examine if mutations of the VHD motif would result in autoactivation of the corresponding MLA proteins. Thus, a histidine to alanine mutation was introduced to MLA1, MLA18-1, MLA25-2 and TmMLA1. The mutants and the corresponding wild-type proteins were transiently overexpressed in *Nicotiana benthamiana* by *Agrobacterium tumefaciens*-mediated transformation (Simon Schwizer, Master Thesis). Overexpression was

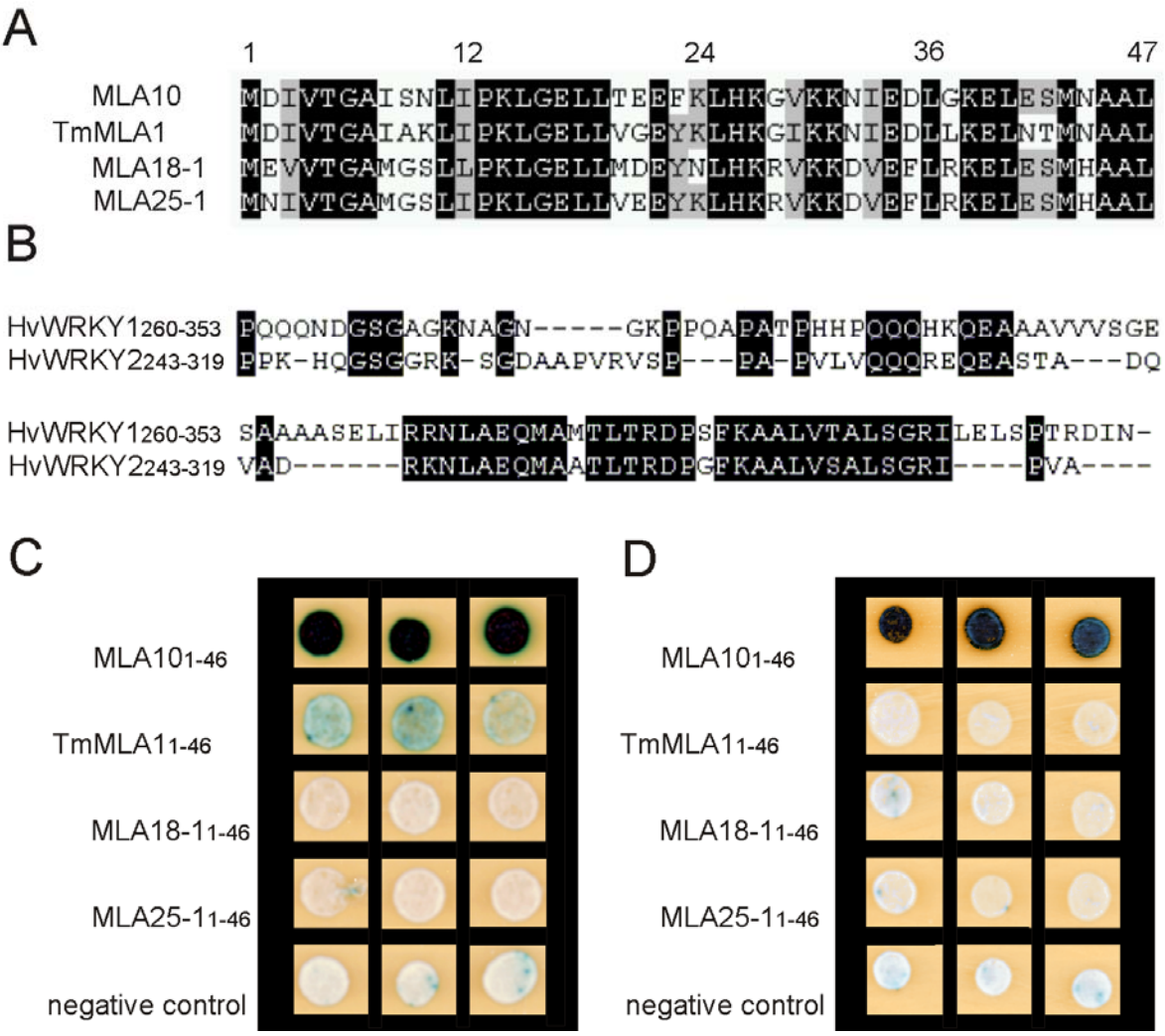


Figure 11: A) Alignment of the first 46 residues of four different MLA CC domains used for yeast two-hybrid interaction tests with the transcription factors HvWRKY1 and HvWRKY2. The MLA10 sequence represents the N-terminus that is 100% conserved in the first 46 residues encoded by 25 validated and candidate MLA cDNAs. The N-termini of MLA18-1 and MLA16-1 are identical. Black: identical amino acid residue, White: polymorphic residue. B) Alignment of the C-termini of HvWRKY1$_{260-353}$ and of HvWRKY2$_{243-319}$ that share a sequence identity of 43%. Both sequences were used for yeast two-hybrid interaction tests with the first 46 residues of the four MLA sequences indicated above. C-D) β-Galactosidase activity of yeast strain EGY48 expressing different fusion proteins. Three different yeast colonies per interaction were grown on media containing X-Gal as substrate. Pictures were taken after 72 h. The assay was repeated after a second independent yeast transformation. C) B42-HvWRKY1$_{260-353}$ tested for interaction against LexA-MLA$_{1-46}$ as a positive control. We observed a weak interaction for LexA-TmMLA1-46, but no interaction for the other two MLA N-termini of MLA18-1 and MLA25-1. D) All four MLA sequences were tested against B42:HvWRKY2$_{260-353}$ with LexA-MLA1$_{1-46}$ as a positive control. Negative control for both experiments: LexA-MLA$_{1-46}$ tested against B42-HvWRKY2$_{178-242}$, the WRKY domain.

driven by an enhanced cauliflower mosaic virus (CaMV) 2x35S promoter. The H501A mutation in MLA1 induced a visible HR in the infiltrated leaf sector (Figure 12). The development of an HR triggered by the autoactive mutant MLA1^{H501A} was visible after two days and resulted in a clearly visible programmed cell death after three days. The mutant protein MLA25-1^{H504A} only triggered a slow response, visible as a weak programmed cell death in the infiltrated sector after three days (Figure 12A). The other two mutants MLA18-1^{H502A} and TmMLA^{H508A} did not induce a visible HR and do not seem to be autoactivating at all. The wild-type proteins MLA1, MLA18-1, MLA25-1 and TmMLA1 showed no programmed cell death during overexpression in *N. benthamiana* after three days (Figure 12). All autoactive mutants and wild-type proteins gave the same results when co-expressed with a gene silencing suppressor (BENDAHMANE *et al.* 2002; VOINNET *et al.* 2003).

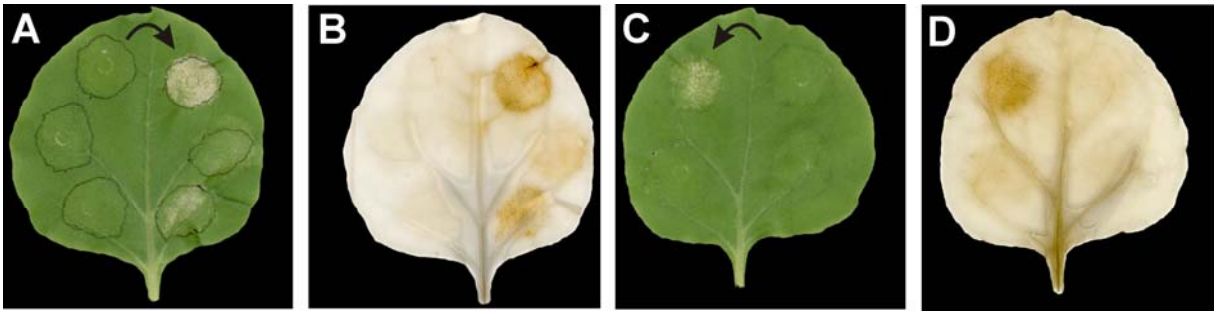


Figure 12: VHD to VAD mutants of four MLA proteins were overexpressed by a 2x35S promoter in leaves of *Nicotiana benthamiana* by agroinfiltration. Pictures of representative leaves were taken three days after infiltration (OD_{600} = 0.1). A) Clockwise: The mutants MLA1^{V501A}, MLA18-1^{V502A}, MLA25-1^{V504A}, MLA25-1 (wt), MLA18-1 (wt) and MLA1 (wt). B) The same leaf as shown in (A), destained in 100% ethanol. C) Counterclockwise: MLA1^{V501A}, TmMLA1^{V508A}, TmMLA1 (wt), MLA1 (wt). D) The same leaf as shown in (C), destained in 100% ethanol. The mutant MLA1^{V501} induced a strong HR resulting in a clearly visible necrosis, whereas only a weak necrosis was observed for MLA25-1^{V504A}. The two mutants MLA18-1^{V502A} and TmMLA1^{V508A} did not show an autoactive phenotype.

It was not possible to compare the spontaneously induced HR triggered by the mutant MLA1^{H501A} with an HR induced by MLA1 and its cognate fungal effector protein in *N. benthamiana* because the latter has not been identified yet. Recently, the two powdery mildew effector genes Avr_{K1} and Avr_{A10} were isolated, postulated to be recognized by *Mlk* and *Mla10*, respectively (RIDOUT *et al.* 2006). Thus, we coexpressed *Mla10* with its suggested cognate effector molecule Avr_{A10} in agroinfiltrated *N. benthamiana* leaves. However, we could not observe a visible programmed cell death in the infiltrated area after few days (for details, see

chapter 4). Thus, no HR response was triggered when MLA10 and AVR_{A10} were coexpressed in *N. benthamiana* under the conditions used. As a positive control, the pepper R protein Bs3 was coexpressed with its cognate effector protein AvrBs3 (ROMER *et al.* 2007), which induced a clearly visible HR after three days (data not shown). For AVR_{A10}, it was only shown indirectly that this fungal effector is recognized by MLA10 (RIDOUT *et al.* 2006) and thus, the recognition has still to be shown by an appropriate assay.

3.3 Discussion

3.3.1. Sequence diversity in the coiled-coil domain and its functional effects

We observed differences between the two domain swap constructs that contained the encoded CC and NB-ARC domain of MLA18-1 cDNA (Swap3) or MLA25-1 cDNA (Swap6) combined with the encoded LRR sequence of MLA1 cDNA. Swap3 was susceptible, whereas Swap6 gave the same strong resistance response as MLA1 when expressed in barley leaves and tested against isolate K1. Thus, the domain swap construct Swap6 demonstrated that MLA25-1 cDNA expression resulted in resistance activity and that it is probable that its own encoded LRR domain is involved in resistance specificity against other powdery mildew isolates than K1 or A6, whereas Swap3 showed that this is not the case for MLA18-1 cDNA. Thus, it is likely that MLA18-1 has only a regulatory function instead of being directly involved in effector recognition. These chimeras additionally confirm that the LRR domain is involved in specificity against different powdery mildew isolates as shown previously for domain swaps between MLA1 and MLA6 (SHEN *et al.* 2003).

MLA18-1 and MLA25-1 share a sequence identity of 90% in the CC domain and 92% in the NB-ARC domain. It is possible that residues that differ in the NB-ARC domains of MLA18-1 and MLA25-1 are important for conferring resistance against isolate K1. Interactions between the NB-ARC and LRR domains were previously demonstrated for the potato R protein Rx (RAIRDAN *et al.* 2008; RAIRDAN and MOFFETT 2006). It is likely that several positions of the NB-ARC domain of MLA18-1 disturb the interaction with the LRR of MLA1, whereas the NB-ARC domain of MLA25-1 contains residues that favour intermolecular folding. The weak HR triggered by expression of mutant MLA25-1^{V504A} in *N. benthamiana* is an additional indication besides the chimera Swap6 that downstream signalling is induced. As no such spontaneously triggered HR was observed for the mutants MLA18-1^{V502A} and TmMLA1^{V508A}, it remains to be investigated if they are directly involved in giving resistance or if they activate yet unknown downstream-regulatory pathways.

In the yeast two-hybrid assay, the N-terminus of the wheat homologue TmMLA1$_{1\text{-}46}$ interacted only with the transcription factor HvWRKY1, but not with HvWRKY2 (Figure 11). TmMLA$_{1\text{-}46}$ differs in nine residues from the sequence of MLA10$_{1\text{-}46}$, in 18 residues from MLA18-1 and in 14 residues from MLA25-1. Thus, the interaction with HvWRKY2 depends on these critical residues. For all MLA variants, the CC domain is predicted to consist of 22 residues, ranging from position 26 to 47 (see chapter 2 and ZHOU *et al.* 2001). It remains to be investigated, which of these 22 positions are important for forming a complex with the WRKY proteins. Neither MLA18-1$_{1\text{-}46}$ nor MLA25-1$_{1\text{-}46}$ showed an interaction with the two WRKY transcription factors HvWRKY1 and HvWRKY2 (Figure 11). However, the two domain swaps of MLA1 with the N-termini of MLA18-1 (Swap2) or MLA25-1 (Swap5) resulted in weak resistance responses when transiently overexpressed in barley (Figure 10). Thus, it is also possible that a third, yet unknown factor interacts with both, MLA and the WRKY transcription factor and stabilizes the protein complex. This stabilization is likely to be more important for weaker interactions as observed between MLA10$_{1\text{-}46}$ and HvWRKY1/2, which would be the case for TmMLA1$_{1\text{-}46}$, MLA18-1$_{1\text{-}46}$ or MLA25-1$_{1\text{-}46}$. If such a factor exists in barley, but not in yeast, it could explain the discrepancy between the yeast two-hybrid interaction tests and the characterisation of chimeras in barley. A second explanation could be that MLA18-1 and MLA25-1 interact with a yet unknown protein, which does not exist in yeast. It is possible that they interact with a different member of the WRKY transcription factor family than HvWRKY1/2. It has been postulated that besides HvWRKY1 and HvWRKY2, two additional WRKY proteins are potential regulators of pathogen response called HvWRKY3 and HvWRKY23 (MANGELSEN *et al.* 2008). Thus, these two proteins are probable interaction partners of MLA18-1 and MLA25-1.

3.3.2 Resistance pathways in monocots compared to the pathways in dicots

The two gain-of-function mutants MLA1^{H501A} and MLA25-1^{H504A} conferred a visible HR when they were expressed in agroinfiltrated *N. benthamiana*. To our knowledge, our work about barley MLA autoactive mutants is the first study on a CC-NB-LRR protein from a monocot overproduced in the dicot *N. benthamiana*. It seems as if the mechanisms that induce the programmed cell death after activation of the CC-NB-LRR immune receptor are similar in monocots and dicots.

To date, all studies about loss-of-function or gain-of-function mutants in R proteins performed in *N. benthamiana* were originally derived from Solanaceous species (VAN OOIJEN *et al.* 2008). There are studies about proteins from *Arabidopsis* or *Medicago truncatula* not being

involved in resistance that were tested in *N. benthamiana* for protein-protein interaction by a bimolecular fluorescent complementation assay (BiFC; reviewed in MESSINESE *et al.* 2007; OHAD *et al.* 2007). Furthermore, the interaction of wheat proteins implicated in abiotic stress response and development that were detected in a yeast two-hybrid screen were confirmed for interaction by the BiFC assay in *N. benthamiana* (TARDIF *et al.* 2007).

There are only three examples of *R* genes not derived from solanaceous species that were transiently expressed in agroinfiltrated *N. benthamiana* leaves. The first example is flax *L6*, which resulted in a weak autoactivation when expressed in *N. benthamiana* from genomic DNA (HOWLES *et al.* 2005). The second example is TIR-NB-LRR gene *RPS4* from *Arabidopsis*, which resulted in a spontaneously triggered HR not visible during expression in *Arabidopsis* itself (ZHANG *et al.* 2004). Interestingly, when *RPS4* was expressed in *N. benthamiana* plants with silenced *SGT1*, HR was not observed (ZHANG *et al.* 2004). The third example is Arabidopsis *RPP13*, which induced HR when coexpressed with its cognate effector gene *ATR13* in agroinfiltrated *N. benthamiana*. Interestingly, *RPP13* did only spontaneously induce a delayed HR, when it was expressed at very high levels (RENTEL *et al.* 2008).

The chaperone HSP90 and the two co-chaperones RAR1 and SGT1 were shown to be required for the stabilization of many R proteins (BOTER *et al.* 2007). The important role of SGT1, Rar1 and Hsp90 seems to be conserved in monocots and dicots (SHIRASU and SCHULZE-LEFERT 2003). This could be an indication that the pathways that are induced to trigger a cell-death response are indeed conserved. We propose that the *N. benthamiana* system is suitable to further investigate these similarities due to many technical advantages in comparison to other plants (GOODIN *et al.* 2008).

As SGT1 is involved in the ubiquitin pathway, it is possible that SGT1 is important for the regulation of both stabilization and degradation of R proteins (BOTER *et al.* 2007). Similar to *RPS4*, the HR induced by coexpression of *Rx*, *Pto* or *Cf-4* with their cognate effector genes were compromised in *SGT1*-silenced *N. benthamiana* plants (PEART *et al.* 2002). Additionally, SGT1 was not required for necrosis based on toxic effects caused by substances like ethanol or sodium azide (PEART *et al.* 2002). Therefore, only a true resistance response would be compromised by down regulation of SGT1, and gain-of-function mutants of R proteins can be tested for a specific HR by expression in *SGT1*-silenced *N. benthamiana*

plants. This was shown for autoactive mutants of Rx, I2 and NRC1 (BENDAHMANE *et al.* 2002; GABRIELS *et al.* 2007; TAMELING *et al.* 2006).
SGT1 was also shown to be important for *Mla* resistance responses in barley (BIERI *et al.* 2004). Therefore, it would be interesting to test if the HR spontaneously induced by the valine to alanine mutants of MLA1 and MLA18-1 depends on SGT1 by expressing them in SGT1-compromised *N. benthamiana* leaves.

Finally, we conclude that MLA25-1 is involved in resistance responses, whereas MLA18-1 seems to have only a regulatory or a completely different, yet unknown, function. It remains to be further investigated what kind of downstream-proteins are activated by MLA18-1 or by MLA25-1 and what kind of role they play in the defence mechanism against powdery mildew.

3.4 Material and methods

3.4.1. Construction of *Mla* domain swaps

To generate the four different domain swaps, the plasmids pENTR-(ubi)-*Mla1*, pENTR-(ubi)-*Mla18-1* and pENTR-(ubi)-*Mla25-1* were used as appropriate PCR templates. Plasmid Swap2 was generated by using the primer pair sse082/sbi295 to amplify the 2735 bp long PCR fragment following the PCR conditions and the PCR programme described for *Pfu*Ultra (Stratagene) in chapter 2.4.2. All PCR reactions were performed in a PTC-200 thermocycler (Bio-Rad). The acceptor pENTR-(ubi)-*Mla18-1* and the amplified fragment were digested using the restriction enzymes *Sac*II and *Not*I (New England Biolabs) and ligated. Plasmid Swap3 was generated by using the forward primer sse071, the reverse primer sbi295, and the overlapping primers sse111 and sse110. A 1665 bp long amplification product was generated by the primer pair sse071/sse110, a 1262 bp long fragment by sse111/sbi295. The same PCR conditions mentioned above were used and both products were purified using the GenElute^{IM} Gel Extraction Kit (Sigma-Aldrich). For the overlap extension PCR (OE-PCR) approach, the amplifications were carried out using the *Pfu*Ultra high-fidelity DNA polymerase (Stratagene). As a template, 200ng of each of the 1665 bp and the 1262 bp long fragments were sufficient. The following amplification conditions were used: 2 min at 94°C, followed by 6 cycles of 20 sec at 94°C, 40 sec at 50°C, 10 min at 68°C. After the 6 cycles, 2 µl sse071 (10 µM) and 2 µl sbi295 (10 µM) were added to the PCR reaction, followed by a second amplification step: 2min at 94°C, followed by 34 cycles of 20 sec at 94°C, 30 sec at 55°C, 2 min at 72°C and then a final extension for 5 min at 72°C. The *Asc*I-*Not*I restriction enzyme pair was used to

digest the 2904 bp long OE-PCR amplification product and the plasmid pENTR-(ubi) before ligation. Swap6 was generated like Swap3 except that forward primer sse073 was used instead of sse071 to generate the 2912 bp long amplification product. Swap5 was generated by using the forward primer sse073, the reverse primer sbi295, and the overlapping primers sse083 and sse084. The 2903 bp long PCR fragment was digested and ligated like Swap3. A C-terminal HA tag was introduced to Swap2 and Swap3 by amplification using the primer pair sse035/sse130 and sse065/sse130 for Swap5 and Swap6. The PCR program described in chapter 2.4.2 was used for all four constructs. The constructs were digested with the restriction enzymes *Asc*I-*Not*I and ligated to the pENTR-(ubi) plasmid. All PCR-generated clones were sequenced using Big Dye Terminator V3.1 following the manufacturer's protocol on an ABI3730 automated sequencer (Applied Biosystems).

3.4.2. Plasmid constructions for yeast two-hybrid interaction tests

The prey fusions pB42AD-WKRY2-d3, pB42AD-WRKY1-CT and pB42AD-WRKY2-CT were obtained from Armin Töller and Paul Schulze-Lefert, MPIZ, Köln (Table 5). The bait fusions were constructed by fusing the 138bp long sequence encoding the 5'end of *Mla10*, *Mla18-1*, *Mla25-1* and *TmMla1* to the LexA binding domain of the pLexA plasmid (Qian-Hua Shen, MPIZ Köln). pLexA-MLA(1-46) carries the N-terminal sequence encoded by *Mla10* (SHEN *et al.* 2007). All bait constructs are listed in Table 5. The 138 bp long 5'end

Table 5: Constructed plasmids for interaction tests

Baits fused to the LexA binding domain

Name	Description
pLexA-MLA18-1(1-46)	MLA18-1_{1-46}/MLA16-1_{1-46}
pLexA-MLA25-1(1-46)	MLA25-1_{1-46}
pLexA-TmMLA1(1-46)	TmMLA1_{1-46}

Preys fused to the B42 activation domain

Name	Description
pB42AD-WRKY2-d3[2]	WRKY domain of WRKY$2_{178-242}$
pB42AD-WRKY1-CT[2]	C-terminus of WRKY$1_{260-353}$
pB42AD-WRKY2-CT[2]	C-terminus of WRKY$2_{243-319}$

[1](SHEN *et al.* 2007), [2]Armin Töller, Paul Schulze-Lefert, MPIZ, Köln

encoding the first 46 residues of *Mla25-1* was amplified by the primer pair sse73/sse072, the corresponding 5'ends of *Mla18-1* with the primer pair sse071/sse072 and the 5'end of *TmMla1* with the primers TJ60/TJ61. PCR amplifications were carried out using the *Pfu*Ultra high-fidelity DNA polymerase (Stratagene) following the manufacturer's manual. The amplification conditions were 2 min at 94°C, followed by 32 cycles of 20 sec at 94°C, 30 sec at 55°C, 60 sec at 72°C, and then a final extension for 5 min at 72°C. All four 146 bp long amplification products were purified using the GenElute^{IM} Gel Extraction Kit and cloned in the bait plasmid. PCR fragments and the vector were digested with the restriction enzymes *BamH*I and *Not*I (New England Biolabs) and ligated.

3.4.3. Construction of autoactive *Mla* mutants

Autoactive mutants of the MHD motif (VHD for MLA) were generated by replacement of the histidine with an alanine (VAN OOIJEN *et al.* 2008). Site-directed mutagenesis was performed by amplification of pENTR-(ubi)-*Mla1* with the primer pair sse143/sse144, pENTR-(ubi)-*gTmMla1* with the primer pair sse145/sse146, pENTR-(ubi)-*Mla18-1* with the primer pair sse149/sse150, and pENTR-(ubi)-*Mla25-1* with the primers sse147/sse148. PCR amplifications were carried out using the *Pfu*Ultra high-fidelity DNA polymerase II (Stratagene) following the manufacturer's manual. The amplification conditions to obtain the PCR products were 2 min at 92°C, followed by 18 cycles of 20 sec at 92°C, 30 sec at 55°C, 5 min at 68°C, and then a final extension for 10 min at 68°C. The generated amplification products were incubated for one hour at 37°C with the restriction enzyme *Dpn*I (New England Biolabs). After the restriction digests, 1 µl per reaction was transformed into one shot Mach1-T1 *E. coli* cells (Invitrogen) following the manufacturer's instructions. The obtained H-to-A mutants and the wild-type plasmids were recombined to the expression vector pMDC32 using the Gateway system (Invitrogen). All pENTR4 plasmids containing inserts flanked by *attL* sites were recombined into the destination vector pMDC32 to create expression constructs. 200ng of the entry clone were mixed with 200 ng of the destination vector, and the volume was completed with TE buffer, pH 8.0 to 4 µl reaction volume. The LR clonase enzyme (Invitrogen, #11791-043) mix was removed from -20°C and thawed on ice for about 2 min. After brief vortexing, 1 µl LR clonase was added to the reaction volume and put back to -20°C storage (very sensitive enzyme). After one hour incubation at 25°C, 1 µl Proteinase K was added, and the mixture was incubated for 10 min at 37°C to terminate the recombination reaction. For transformation, 2 µl of the LR reaction were added to 50 µl $RbCl_2$ competent

DH5α cells. After incubation for 30 min on ice, the cells were heat shocked for 30 sec at 42°C. After 5 min on ice, 250 µl LB medium was added and the cells were plated on LB agar plates after an incubation time of one hour at 37°C. All entry clones were modified by the insertion of an ubiquitin promoter (see chapter 2). After recombination to pMDC32, the plasmids were digested with the restriction enzyme *Asc*I (New England Biolabs) to get rid of the ubiquitin promoter. After purification by the GenElute^{IM} Gel Extraction Kit, the plasmid was ligated. All plasmids used for overexpression in *N. benthamiana* are listed in Table 6.

Table 6: Expression plasmids for agroinfiltration in *N. benthamiana*

Entry clone (expression in barley)	**destination clone (expression in *N. benthamiana*)**	**description**
pENTR-(ubi)-*Mla1*	pMDC32-*Mla1*	cDNA of *Mla1*
pENTR-(ubi)-*Mla18-1*	pMDC32-*Mla18-1*	cDNA of *Mla18-1*
pENTR-(ubi)-*Mla25-1*	pMDC32-*Mla25-1*	cDNA of *Mla25-1*
pENTR-(ubi)-g*TmMla1*	pMDC32-*gTmMla1*	gDNA of *TmMla1*
pENTR-ubi-*Mla1*^{H501A}	pMDC32-*Mla1* ^{H501A}	cDNA of *Mla1*, VHD to VAD mutation
pENTR-ubi-*Mla18-1* ^{H502A}	pMDC32-*Mla18-1* ^{H502A}	cDNA of *Mla18-1*, VHD to VAD mutation
pENTR-ubi-*Mla25-1* ^{H504A}	pMDC32-*Mla25-1* ^{H504A}	cDNA of *Mla25-1*, VHD to VAD mutation
pENTR-ubi-g*TmMla1* ^{H508A}	pMDC32-g*TmMla*1 ^{H508A}	gDNA of *TmMla1*, VHD to VAD mutation

3.4.4. Yeast two-hybrid assay

Yeast strain EGY48 harbouring the *lacZ* reporter gene on the autonomous plasmid p8op-LacZ was transformed with appropriate prey and bait plasmid DNA by the lithium acetate method (GIETZ and WOODS 1998). For yeast transformation, 50 ml SD medium (see chapter 6.4) were inoculated with 3 EGY48 colonies resuspended in 50 µl SD medium and grown at 200 rpm overnight at 30°C. The OD_{600} was adjusted to 0.2 with SD medium. After obtaining an OD_{600} of 0.6, the culture was centrifuged at 2000 rpm for 5 min using a Table centrifuge (Megafuge 1.0, Kendro). The pellet was resuspended in 2.5 ml and stored at room temperature until usage. For 10 transformations, a PEG/LiOAc master mix was prepared (2.4 ml 50% PEG (sterile), 360 µl LiOAc (1 M, sterile filtrated) and 250 µl single-stranded carrier DNA (prepared as described in (Yeast Protocols Handbook, Clontech). For each reaction, 300 µl PEG/LiOAc

was added to the DNA (1.5 µg of each plasmid) and briefly vortexed. Then, 100 µl of yeast cells were added to each reaction and the cell suspension was vortexed for 1 min. The cells were incubated in a water bath at 42°C for 45 min. After incubation, the cells were centrifuged for 5 min at 2500 rpm in a small table top centrifuge and the pellet was dissolved in 150 µl 0.9% NaCl (sterile). The transformed yeast cells were grown on SD plates (see chapter 6.4) for 3 to 4 days at 30°C. Interaction analyses were done by the agar plate assay. No interaction was observed for all four bait constructs when tested against the WRKY domain of HvWRKY2 as a prey. Thus, the interaction between the WRKY domain only and $MLA10_{1\text{-}46}$ was used as a negative control. For each tested interaction, three different yeast colonies from the transformation plate were streaked out on SD plates and grown for 48 h at 30°C. Yeast colonies used for the *in vivo* agar plate assay should not be older than 14 days. Each of the three yeast colonies was resuspended in 50 µl SD medium to inoculate 4 ml of SD medium. These liquid cultures were grown at 30°C overnight. For each liquid culture, the OD_{600} was adjusted with water to 0.6. 3 µl of each culture were applied to an SD X-Gal plate (see chapter 6.4) and dried completely. The selection plates were incubated at 30°C for 72 to 96 h.

3.4.5. Transformation of *A. tumefaciens*

1.0 µl plasmid was added to 40 µl frozen electrocompetent *A. tumefaciens* cells. The suspension was thawed and transferred to the cooled electrocuvette. Following conditions were used for the transfomation: 1.25 volts, 125 µFD and 400 Ohm resistance. After pulsing, 250 µl LB medium were added to the cells. The cells were incubated at 28°C for 2-3 h. Finally, the cells were plated on selective media and incubated for 48 h at 28°C. For *A. tumefaciens* strain GV3101 (HOLSTERS *et al.* 1980), 50 µg/ml rifampicin (stock: 50 mg/ml in DMSO, stored at -20°C) was added to the medium. For Ti-plasmid pMP90 selection, 25 µg/ml gentamicin (stock: 25 mg/ml in water, stored at 4°C) was used plus a third antibiotic selection of the plasmid of interest.

3.4.6. Infiltration of *N. benthamiana* by *A. tumefaciens*

N. benthamiana plants were grown in the greenhouse under standard conditions for three weeks (16 h of light, 25°C and 80% humidity). The efficiency of infiltration is lower after flowering. For the preparation of the bacterial cultures, 4 ml LB medium supplemented with the required antibiotics were inoculated with strain GV3101 harbouring the plasmid of

interest at 200 rpm for 24 h at 28°C. For this purpose, the picked colony was resuspended in 500 µl LB medium to avoid a clumpy suspension. The cultures were centrifuged at 4000 rpm (Eppendorf centrifuge) for 15 minutes at RT. The pellets were resuspended in 1 ml AS medium (3 M $MgCl_2$ (autoclaved), 1 M MES-KOH buffer (sterile-filtrated, pH 5.6) and 150mM acetosyringone (solved in DMSO, stored at -20 °C, Sigma-Aldrich D134406). The OD_{600} was adjusted to 0.7-0.8 with AS medium for each culture. If GV3101 strains with different plasmids were mixed, they were incubated in a ratio of 1:1 for 4 h at RT. For experiments performed with the gene silencing suppressor p19, the MLA constructs in GV301 were mixed with *A. tumefaciens* strain C58C1, carrying the p19 silencing suppressor from tomato bushy stunt virus (VOINNET *et al.* 2003) in a ratio 1:1 to obtain OD_{600} of 0.7. The three-week old *N. benthamiana* plants were watered sufficiently before inoculation. Then, the mixture was injected into the lower side of several fully expanded leaves with a blunt syringe and the infiltrated area was marked with a pencil. After infiltration, the plants were incubated in the greenhouse for 12 hours in the dark and afterwards under the same conditions mentioned above.

3.4.7. Single-cell transient expression assay

The single-cell transient expression assay to test Swap2, Swap3, Swap5 and Swap6 for a resistance response against powdery mildew isolates Bgh A6 and Bgh K1 using *Mla1* and *Mla*6 as controls was carried out as described in chapter 2.4.3. For all experiments, barley plants or detached leaves with or without fungal spores were kept at 20°C, 70% relative humidity and 16 h light/8 h dark cycle. The *Blumeria graminis* f. sp. *hordei* strains A6 and K1 were maintained on barley cultivars Pallas P01 and Pallas P03, respectively.

4. Experimental approaches to establish a simple assay for the study of AVR_{A10}/AVR_{K1} recognition

4.1 Introduction

The resistance gene *Mla* exists as a large series of 29 isolated sequences designated as validated and candidate MLA cDNAs (described in chapter 2) that are highly diverse in the encoded LRR domain. Furthermore, two powdery mildew effector genes AVR_{A10} and AVR_{K1} that are thought to be recognized by *Mla10* and *Mlk1*, respectively, were recently isolated (RIDOUT *et al.* 2006). *Mlk1* is a powdery mildew resistance gene not yet isolated at the molecular level.

There are only few functional assays to test alleles of effectors together with alleles of the cognate *R* gene. For *Arabidopsis RPP13*, alleles of the cognate effector *ATR13* were transiently expressed in *Arabidopsis* lines containing a specific *RPP13* allele (ALLEN *et al.* 2004). Recently, an efficient system was established to investigate the *RPP13-ATR13* recognition by delivery of the cognate *ATR13* by *Pseudomonas syringae pv. tomato* bacteria into *Arabidopsis* lines harbouring the corresponding *RPP13* allele. ATR13 was fused to a signal peptide that allows for the translocation of the protein into the host *Arabidopsis,* resulting in a defence response (RENTEL *et al.* 2008; SOHN *et al.* 2007). In another similar assay, *ATR13* was transferred into the single-stranded RNA genome of the turnip mosaic virus (RENTEL *et al.* 2008). *Arabidopsis* lines containing an *RPP13* allele that recognized the virus-delivered *ATR13* expression showed a clearly visible defence response. Finally, the authors also demonstrated that *RPP13* induces HR when co-expressed with its cognate effector *ATR13* in *Nicotiana benthamiana* by infiltration with *Agrobacterium tumefaciens* (RENTEL *et al.* 2008).

Several other R proteins were produced together with their cognate effectors in *N. benthamiana,* which induced a visible HR in the infiltrated area. This was shown for pepper Bs3/AvrBs3, potato Rx/PVX coat protein, tobacco N/P50, tomato Bs4/AvrBs4, tomato Cf-4/Avr4 or Avr3, tomato Cf-9/Avr9 and tomato Pto/AvrPto (MESTRE and BAULCOMBE 2006; PEART *et al.* 2002; ROMER *et al.* 2007; SCHORNACK *et al.* 2004).

In this study, we wanted to develop an assay to test all validated and candidate MLA cDNAs against alleles of their cognate effector gene. We tested two different approaches, either

transient expression of the effector gene in different barley cultivars by infection with barley stripe mosaic virus (BSMV) or coexpression of *Mla* and AVR_A genes in agroinfiltrated *N. benthamiana* leaves (Simon Schwizer, Master thesis).

4.2 Results and discussion

4.2.1. Design of different BSMV constructs

The barley stripe mosaic virus (BSMV) has a single-stranded positive-sense RNA genome divided into three different components. The architecture of the three unique RNA species, the α, β and γ subunits and their encoded proteins are shown in Figure 13A (LAWRENCE and JACKSON 2001). Each RNA carries a 5'-terminal cap structure and a 3'-terminal tRNA-like structure (PETTY *et al.* 1989). Viral replication depends on α and γ RNAs and viral infection (local and systemic) on RNAβ. A replicase protein with a helicase domain, αa, is encoded by RNAα. A second replicase protein, γa, contains a polymerase motif and is encoded by the RNAγ. The second gene (γb) of RNAγ is thought to be involved in expression regulation of proteins encoded by RNAβ. BSMV RNAβ encodes five different proteins, including the viral coat protein encoded by the gene βa. Three overlapping genes termed the "triple gene block" are present adjacent to βa. These genes, βb, βc, βd and βd' were shown to be important for movement of the virus to cause systemic infection (LAWRENCE and JACKSON 2001).

Virus-induced gene silencing (VIGS) has been established as a tool for functional genomics, where RNA silencing of a gene of interest is achieved by transient expression of homologous gene fragments carried by the viral RNA (HOLZBERG *et al.* 2002). In barley and wheat, VIGS based on BSMV was shown to be a useful tool for the silencing of genes associated with powdery mildew or leaf rust resistance (HEIN *et al.* 2005; SCOFIELD *et al.* 2005; SHEN *et al.* 2007).

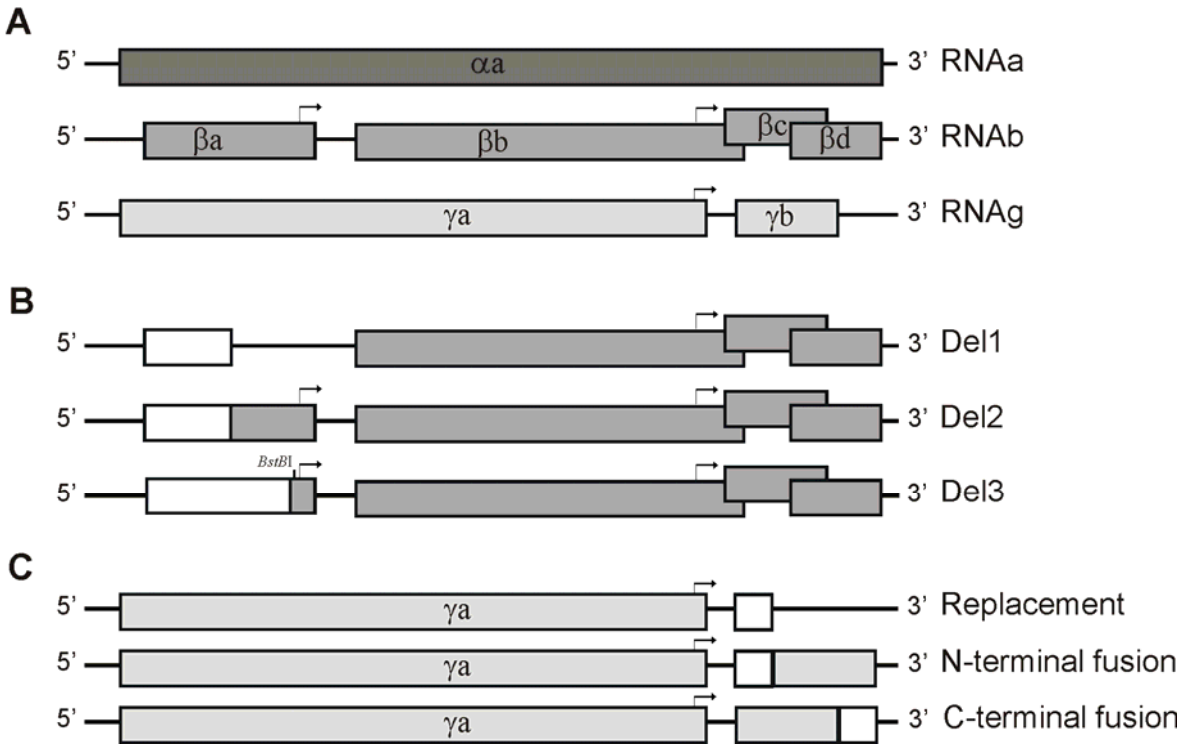


Figure 13: Illustration of the genomic organization of barley stripe mosaic virus (BSMV) and the design of expression constructs (The illustrations are based on HOLZBERG *et al.* 2002). Dark grey: RNAα, grey: RNAβ, light grey: RNAγ, white: designed cloning cassettes A) The three subgenomes of BSMV with the indicated viral genes. B) The three constructs designed by modifications of the coat protein βa. White: cloning cassette. C) The three constructs designed by modifications of the γb protein.

The advantages of using a virus as a tool for studying gene expression instead of gene silencing lead us to the idea of establishing an assay called virus-induced gene expression (VIGE). The three BSMV subgenomes were modified to develop suitable expression constructs of the two powdery mildew effectors AVR_{A10} and AVR_{K1} (for details see chapter 4.3). We wanted to investigate whether their expression using VIGE in barely cultivars harbouring different *Mla* alleles would result in recognition responses. It was shown previously that GFP is expressed in infected barley tissue when fused C-terminally to the BSMV γb protein (LAWRENCE and JACKSON 2001). First, we intended to express the YFP gene as a positive control to assess the point where expression of the fusion constructs reaches a high level. Then, in a second step, the aim was to express the powdery mildew effectors AVR_{A10} and AVR_{K1} (RIDOUT *et al.* 2006). As a positive control, a barley cultivar with *Mla10* would be infected with a viral AVR_{A10} construct, which should result in a clearly visible defence response on the virus-infected plants. In contrast, only virally induced infection

symptoms should be visible for the negative control with AVR_{K1} in *Mla10*-containing plants. Finally, we wanted to screen different barley cultivars that either harbour an already isolated *Mla* allele or were described to contain a certain *Mla* specificity for AVR_{A10} or AVR_{K1} recognition.

For BSMV, it was shown that the RNAβ is important for infection of whole plants, but not for the infection of barley protoplasts (PETTY and JACKSON 1990). Several deletion mutations were introduced into the "triple gene block" (βb, βc, βd and βd') that eliminated infectivity of the RNA. Furthermore, deletion of the coat protein encoded by βa showed that it is not essential for BSMV multiplication and systemic movement *in planta* (PETTY and JACKSON 1990). Part of the region encoding the coat protein was shown to be involved in triple gene block regulation and several mutations within the coat protein affected the expression of the downstream triple gene block and also the stability of the viral RNA (PETTY and JACKSON 1990).

Based on this knowledge, different constructs were designed for the β subgenome by the insertion of a cloning cassette (Figure 13B). As all three BSMV subgenomes were carried by a pBluescript vector, the corresponding plasmids were modified for the constructs listed in Table 7 (for details see chapter 4.3). One of our designed constructs was a complete deletion of βa termed Del1. A similar construct was shown previously to have no influence on virally induced systemic infections (PETTY and JACKSON 1990). As the region of the βa gene sequence that is necessary for triple gene block regulation is not exactly known, another construct was designed where the first half of the βa gene was replaced by a cloning cassette, called Del2. In a third construct, Del3, βa was truncated as done previously to silence the phytoene desaturase (PDS; HOLZBERG *et al.* 2002). The three cloning cassettes (Del1, Del2 and Del3) were designed to express YFP, AVR_{A10} and AVR_{K1}, and constructs were made as listed in Table 7.

As in the γb-GFP fusion mentioned above, most gene silencing constructs were C-terminally fused to the γb protein (HOLZBERG *et al.* 2002; SHEN *et al.* 2007). A study accomplished silencing by inserting part of a gene of interest at the beginning of the γb gene, preventing the expression of γb by a truncated start codon (TAI *et al.* 2005). Based on these studies, we designed cloning cassettes fused the γb gene either on the 5'- or 3'-end (Figure 13C). A third

construct was a complete replacement of the γ-b by a cloning cassette, although it was not known if the virus is still functional without this protein. All expression constructs involving the γ-b gene created for YFP are listed in Table 7.

4.2.2. Infection with different BSMV constructs

Silencing of the phytoene desaturase (PDS) coding gene results in photo-bleaching of virally infected barley (HOLZBERG *et al.* 2002). This easily visible phenotype was induced by the construct pγb:PDS-as (Figure 14A), and was taken as positive control for all performed VIGE experiments. In a first step, we wanted to test if BSMV variants harbouring the designed RNA constructs encoding a modified coat protein or γ-b protein (Table 7) do not influence virally induced systemic infection. We intended to test only the constructs for YFP expression, which showed BSMV infection without carrying an insert and thus, having no negative influence on the virus. The constructs pβ:Del1 (Figure 14B), pβ:Del2 (data not shown) and pγ:C (Figure 14C) of these BSMV variants caused systemic infection in barley after 10 days. No virally induced systemic infection symptoms were visible when the construct pγ:R was *in vitro* transcribed as RNAγ (data not shown). Nevertheless, the characteristic necrotic spots on the first leaf, the site of viral infection, were also observed for pγ:R and seem to be a virally induced local infection, which do not result in systemic infection.

We wanted to find the time point, where the expression levels of the BSMV fusion constructs are high. Thus, we exploited VIGE for the expression of *YFP* in the *Mla*-negative barley cultivar Morex as a positive control. First, constructs expressing *YFP* were tested for virally induced infection symptoms (Table 7 and Figure 14D). Instead of a systemic infection, necrotic spots were observed on the first leaf for pβ:Del2-YFP and pγ:R-YFP as described for pγ:R (data not shown). Thus, these constructs seem to be non-functional in systemic infection. Results were more different for plants inoculated with pβ:Del1-YFP (Figure 14D) and pγ:C-YFP (data not shown). There, both constructs induced a systemic infection after 13 days. Infection was slightly faster for plants inoculated with the constructs without YFP, where the virally induced symptoms were already visible after 10 days. We tried to find the time point

Table 7: VIGE constructs
Summary of the constructs made for virus-induced gene expression (VIGE) with the three plasmids carrying the three subgenomes of BSMV.

Construct	Expression	Viral symptoms
pβ:Del1	replacement of half of the viral coat protein with a cloning cassette	visible
pβ:Del2	truncated version of the viral coat protein containing a cloning cassette	visible
pβ:Del3	replacement of the viral coat protein with a cloning cassette	not investigated
pγ:N	N-terminal g-b fusion cassette	not investigated
pγ:C	C-terminal g-b fusion cassette	visible
pγ:R	Replacement of g-b with a cloning cassette	only local infection on the first leaf
pβ:Del1-YFP	YFP expression	visible
pβ:Del2-YFP	YFP expression	only local infection on the first leaf
p:βDel2-A10	Avr_{A10} expression	not investigated
p:βDel2-K1	Avr_{K1} expression	not investigated
pγ:C-YFP	YFP expression	not investigated
p:γR-YFP	YFP expression	only local infection on the first leaf

All three subgenomes are in pBluescript vectors including the corresponding constructs. Viral symptoms were classified as visible and systemic when the characteristic stripes occurred on the 3rd, 4th or 5th leaf ten days after BSMV infection. Local infections were observed on the first leaf, which was infected with BSMV, as necrotic spots. Not investigated means that this construct has not been tested for viral symptoms.

with maximal YFP expression. 10 days after BSMV infection, the second, third and fourth leaves of plants expressing Del1-YFP were investigated for YFP expression by a fluorescence microscope. As a negative control, pβ:Del1 without YFP was used (Figure 14E). After 10 days, YFP expression was not clearly distinguishable from the fluorescent background visible in the negative control (Figure 14F). As a positive control, YFP was transiently overexpressed in barley cultivar Morex (Figure 14G). It remains to be further investigated at which time point most effective expression would be observed. Additionally, it would be necessary to test the presence of YFP by immunoblot analysis, due to the autofluorescent background.

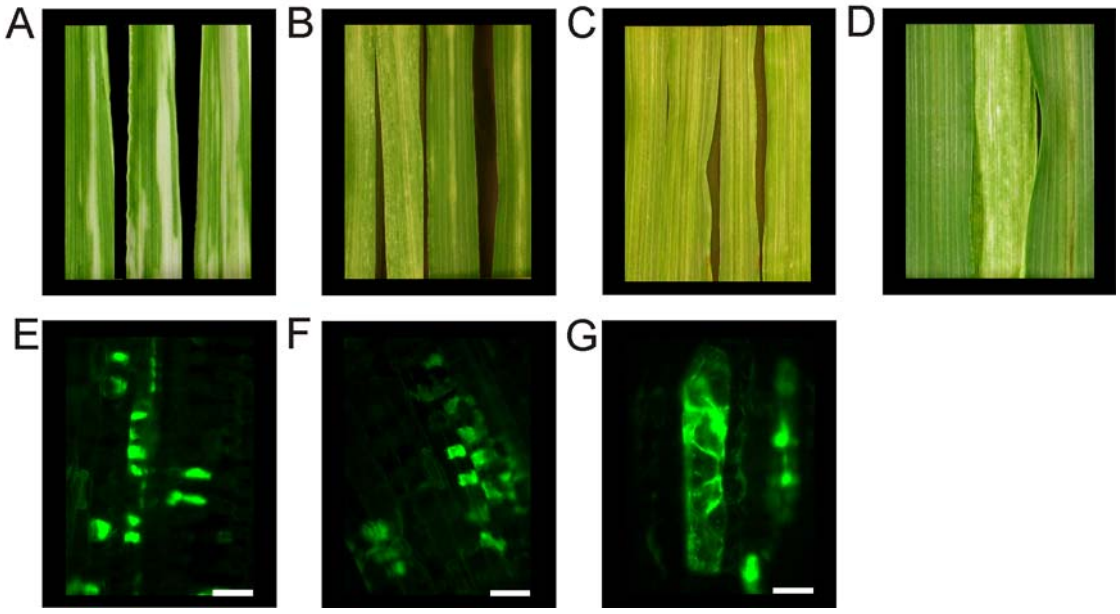


Figure 14: Viral-induced expression of modified β or γ BSMV subgenome constructs. A-D: Systemic infection symptoms of BSMV infected plants of barley cultivar Morex. Pictures were taken 10 days after infection of the third leaves of infected plants. Contrast and brightness of images were altered in CorelDraw X3. A) PDS silencing in barley cultivar Nigrate with the pγ:PDSas construct was used as a positive control. The silencing resulted in clearly visible BSMV infection symptoms. B) Leaves were inoculated with viral particles containing besides RNAα and RNAγ, pβ:Del1 instead of RNAβ. The expression of pβ:Del1 resulted in visible systemic infection symptoms. C) The expression of pγC instead of RNAγ resulted in clearly visible virus-induced symptoms. D) The expression of YFP delivered by the RNAβ of pβ:Del1-YFP resulted in visible systemic infection. E-G) Fluorescence was observed 10 days after viral infection by epifluorescence microscope. Only the third leaves containing virus-induced symptoms were investigated. A GFP filter was used to detect YFP fluorescence. E) Morex plants infected with BSMV harbouring the construct pβ:Del1 without YFP were used as a negative control. F) The expression of YFP was not differentiable from the background fluorescence of the cells induced to viral infection. G) Transiently overexpressed full-length YFP in barley cultivar Morex was used as a positive control. Scale bars represent 50 μm.

It was shown previously that *GFP* was expressed only in protoplasts and not in epidermal cells when C-terminally fused to the γb protein (LAWRENCE and JACKSON 2001). In this case, *GFP* expression was already observed after 96 h post inoculation in systemically infected leaves (LAWRENCE and JACKSON 2001). These authors could even detect *GFP* expression throughout most of the chlorotic regions, indicating that the GFP fluorescence is clearly different from background fluorescence. Thus, the BSMV-infected barley plants expressing *YFP* should also be examined at an earlier time point, even if no viral symptoms are visible, for example after 96 h as described for *GFP* (LAWRENCE and JACKSON 2001). It is possible that examining the fluorescence using an YFP filter instead of a GFP filter could improve the discrimination from the fluorescent background.

The pβ:Del2-YFP construct did not show any viral infection, in contrast to pβ:Del2 without YFP. Coat protein mutants seem to be unstable according to LAWRENCE and JACKSON (2001). These authors mentioned that derivatives containing a GUS substitution for the coat protein βa failed to replicate and that the reporter gene was deleted in virus recovered from infected plants (LAWRENCE and JACKSON 2001). Thus, it is possible that the cloned constructs are unstable and therefore non-functional. Immunoblot analysis should be conducted to determine whether the coat replacement constructs as well as the γb fusion construct are stable during BSMV induced systemic infection.

As mentioned earlier, the establishment of a suitable YFP expression construct should lead to an assay where the expression of the two powdery mildew effector molecules AVR_{A10} and AVR_{K1} encoded by BSMV would be possible. With this assay, several barley cultivars harbouring a known *Mla* gene would be tested for either recognition of AVR_{A10} or AVR_{K1}. The identification of functional *Mla* genes by BSMV-mediated VIGE of powdery mildew effectors is difficult because most barley cultivars including Ingrid- or Pallas-derived near-isogenic lines are resistant against BSMV. This is not the case for Manchuria-derived near-isogenic lines, from which five *Mla* genes were isolated (see chapter 2). On the other hand, six alleles were isolated from near-isogenic Ingrid lines. Additionally, most candidate MLA cDNAs were even isolated from landraces or wild barley-derived cultivars, where no isogenic *Mla* lines are available. Some of these cultivars contain more than one *Mla* gene or other powdery mildew resistance genes like *Mlk* (see chapter 2). Here, results from the VIGE assay could be difficult to interpret, because the observed phenotype cannot be unambiguously attributed to a specific *Mla* gene.

Thus, the approach to test *Mla* – AVR_A specificity using BSMV as an expression vector of the corresponding effector molecules has some problems. However, the fact that effector recognition by different *Mla* genes could be performed in the original host plant could also be an advantage of this assay because the signalling cascade that leads to a defence response would be present. As mentioned above, VIGE was already successfully established for expressing *ATR13* alleles in *Arabidopsis* plants containing certain *RPP13* alleles. Virus-induced systemic infection resulted in visible mottling symptoms on young rosette leaves when a certain *ATR13* allele was recognized. The mottling symptoms were observed in the same area where expression of the GFP gene alone induced by VIGE was visible (RENTEL *et al.* 2008).

Finally, the difficulties of establishing VIGE and the lack of sufficient isogenic barley cultivars that are BSMV susceptible lead us to change to a potentially more suitable assay. Optimally, such an assay would combine several advantages: First, it should be easy in handling and suitable to screen a large number of potential interactions. Second, it should eliminate the possibility that the effector is recognized by something else than the tested *Mla* gene. With these criteria in mind, we changed to *Agrobacterium tumefaciens*-induced co-infiltration of *Nicotiana benthamiana* to examine the recognition between MLA and AVR_A proteins. This method has been successfully applied to pepper *Bs3* and its cognate effector *AvrBs3* (ROMER *et al.* 2007).

4.2.3. Attempts to establish an *Mla* recognition assay in *N. benthamiana*

To perform the expression of all 28 isolated *Mla* sequences (note that *Mla38-1* was omitted) in *N. benthamiana* together with either AVR_{A10} or AVR_{K1}, we wanted to design suitable expression constructs. All 28 validated and candidate MLA cDNAs were previously cloned into a modified entry vector containing an ubiquitin promoter for overexpression in barley (see chapter 2). We intended to use a slightly modified protocol (see chapter 4.3.) of the gateway system (Invitrogen) to clone all of these cDNAs into the plant transformation vector pMDC32 (CURTIS and GROSSNIKLAUS 2003) featuring an enhanced version the Cauliflower mosaic virus (CaMV) 35S promoter.

In a co-infiltration experiment, *Agrobacterium tumefaciens* liquid cultures containing different plasmids of interest were mixed and injected into leaves of *Nicotiana benthamiana* using a blunt syringe (Simon Schwizer, Master thesis). In this approach, one culture contained one of the 28 MLA cDNAs in the appropriate expression plasmid and the other culture carried either AVR_{A10} or AVR_{K1}. If the produced AVR is recognized by the corresponding MLA protein, this would lead to an HR that is visible on the site of infiltration. As a positive control, the pepper resistance gene *Bs3* and its cognate effector *AvrBs3* from *Xanthomonas campestris pv. vesicatoria* were used (ROMER *et al.* 2007). AvrBs3 recognition by Bs3 triggered a visible HR after three to four days (Figure 15). In contrast, no signs of programmed cell death were observed when *Mla10* was coexpressed with either AVR_{A10} or AVR_{K1} (Figure 15).

Several reasons could explain this lack of triggered HR concerning the *Mla10*-AVR_{A10} coexpression. It is possible that the plant defence mechanisms are different in the monocot plant barley compared to the dicot *N. benthamiana*. For example, the signalling cascades or transcriptional activation could be very different. However, the autoactive MLA1 mutant that spontaneously triggered HR in *N. benthamiana* (see chapter 3) indicates that both monocots and dicots share similar pathways. Additionally, SGT1 also occurs in *N. benthamiana,* and it was shown that SGT1 together with the chaperone HSP90 and the co-chaperone RAR1 are important for R protein stabilization (AZEVEDO *et al.* 2006).

A second explanation could be that the proteins are not expressed in *N. benthamiana*. To investigate this assumption, *Mla10* was cloned with an HA tag or with an *YFP* gene fused at the 3'-end. For MLA expression constructs, it is known that N-terminal fusions are non-functional, but not C-terminal fusions (BIERI *et al.* 2004). As nothing is known about the functions of the two effector genes AVR_{A10} and AVR_{K1}, (RIDOUT *et al.* 2006), we tried to detect the gene products by immunoblotting. For this purpose, these two genes were cloned with a myc tag fused either to the 5'- or 3'-end. Attempts to confirm the expression of *Mla10* and the two effector genes by immunoblot analysis were unsuccessful. This kind of analysis has to be further improved to obtain satisfying results. Still, it was possible to detect the expression of the *Mla10-YFP* fusion construct in *N. benthamiana* (Simon Schwizer, Master thesis). The fluorescence was examined two days post-infiltration (Figure 16). Thus, the resistance gene *Mla10* is expressed in *N. benthamiana*.

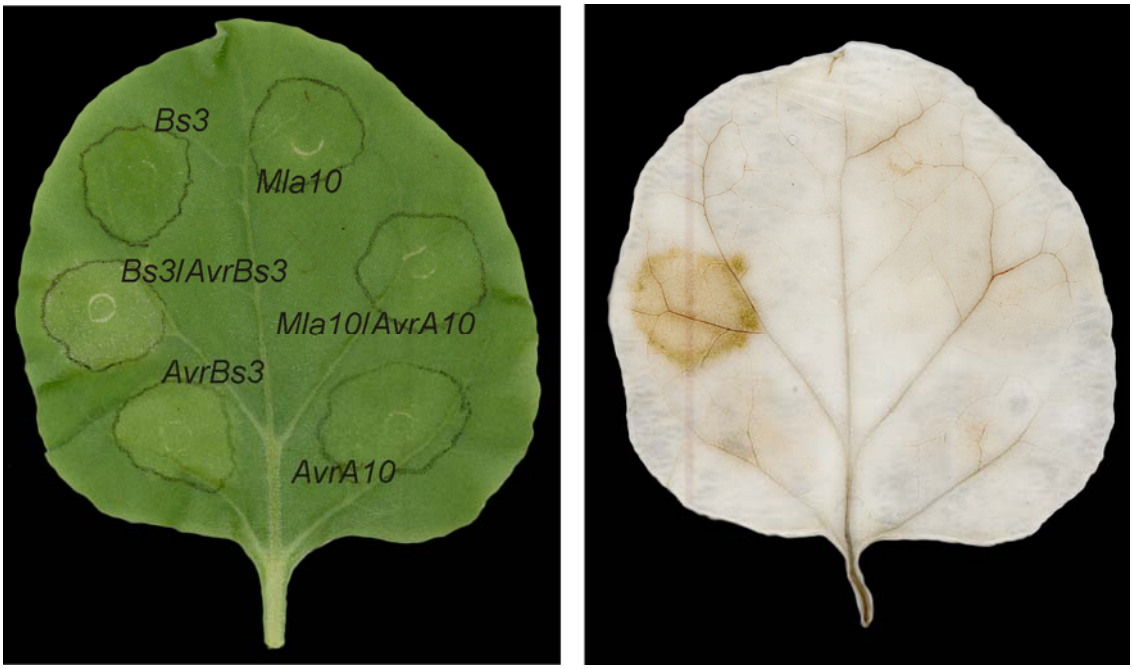


Figure 15: Infiltration of *Nicotiana benthamiana* leaves with *Agrobacterium* transformed with different constructs. Left: *Bs3* triggered a visible HR after four days when expressed together with *AvrBs3*. No HR was observed while expressing the resistance genes *Bs3* or *Mla10* without effector and for *Mla10* expressed together with *AvrA10*. Right: The same leaf as on the left site destained in 100% ethanol to visualize the programmed cell death triggered in the infiltrated region of *Bs3*/*AvrBs3*.

Little is known about the two powdery mildew effectors AVR_{A10} and AVR_{K1} and their function (RIDOUT *et al.* 2006). Thus, a third explanation for the lack of a visible HR by *Mla10*-AVR_{A10} coexpression could be that these effectors do not induce the HR response. To test if the isolated effectors are recognized by the cognate resistance proteins expressed by *Mla10* or *Mlk*, a cell-death assay was used after transient expression of the effectors in the near-isogenic barley cultivars Pallas P09 (*Mla10*) and Pallas P17 (*Mlk*). Thus, the authors showed only indirectly that AVR_{A10} is recognized by MLA10 and AVR_{K1} by MLK (RIDOUT *et al.* 2006). It remains to be investigated how AVR_{A10} is recognized by MLA10 in more detail. For example by using a functional assay where both proteins are expressed together in an appropriate background to exclude the involvement of other *Mla* genes. As the transient coexpression of *Mla10* and AVR_{A10} did not trigger HR in *N. benthamiana*, it is likely that an assay involving the host plant barley is more appropriate. Nevertheless, the two isolated powdery mildew genes seem to be involved in virulence, as a defence answer is observed in the cell-death assay (RIDOUT *et al.* 2006).

Thus, it remains to be investigated whether AVR_{A10} is the true cognate effector recognized (directly or indirectly) by MLA10. As mentioned above, AVR_{A10} recognition was demonstrated by an indirect assay using the near-isogenic barley cultivar Pallas P17, which harbours the unknown powdery milew resistance gene *Mlk* (RIDOUT *et al.* 2006). This near-isogenic barley cultivar Pallas P17 obtained the characterised gene *Mlk* from barley cultivar Monte Cristo. Interestingly, the same barley cultivar Monte Cristo was used by breeders as parent line for the *Mla9* specificity in the near-isogenic line Pallas P07. Previously, it was shown that *Mlk* is closely linked to the *Mla* locus (GIESE *et al.* 1981). Thus, it could be possible that the effector molecule assigned as AVR_{K1} is in fact AVR_{A9}. Therefore, we isolated the MLA9 cDNA sequence from Pallas P17 and Pallas P07. Sequencing of the 5'- and 3'-ends of the isolated *Mla* full-length cDNAs showed that both lines indeed contain the candidate MLA9 cDNA.

Finally, we believe that co-expression of a resistance gene together with its cognate effector molecule would be an elegant assay for the investigation of recognition specificity. In case of the 29 MLA cDNAs that are highly polymorphic in the encoded LRR domain, such an assay could greatly contribute to answer the question why *Mla* genes are so diverse in sequence.

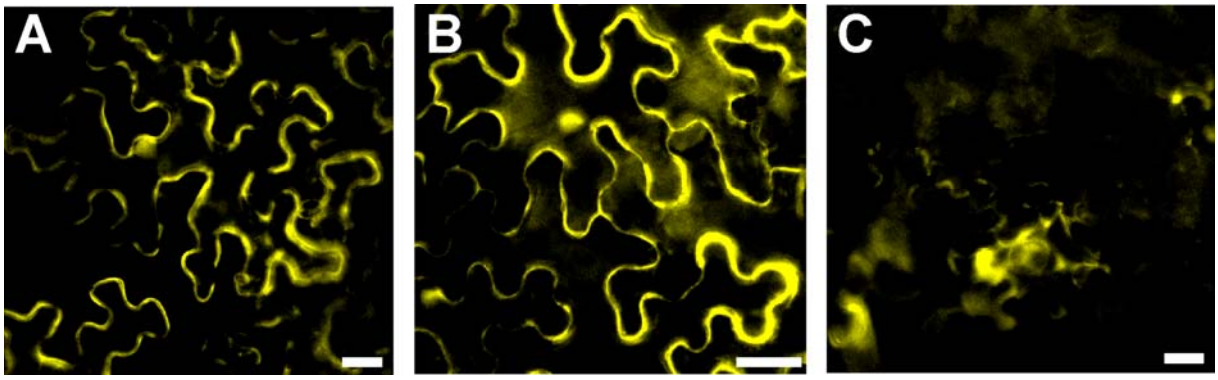


Figure 16: Agrobacterial infiltration of *N. benthamiana.* Fluorescence was investigated three days after infiltration using an epifluorescence microscope equipped with an YFP filter. False colours are shown. A) *Mla10-YFP* expression in the infiltrated region. B) The expression of the tonoplast-localized construct *TT12-YFP* was used as a positive control. C) fluorescence background in the infiltrated region expressing *Mla1* without a YFP tag. Scale bars represents 20 µm.

4.3 Materials and methods

4.3.1. General materials

The *Mla*-negative barley cultivar Morex (CIho 15773; WEI *et al.* 2002) used for VIGS and VIGE studies was derived from seeds obtained from the stock centre USDA National Small Grains Research Facility (USA) and Nigrate (HOR 14775), Pallas P07 (Monte Cristo (*Mla9*), NGB 4937) and Pallas P17 (Monte Cristo (*Mlk*), NGB4948) from IPK Gatersleben (Germany). For all experiments, plant material or detached leaves were kept at 20°C, 70% relative humidity and 16 h light/8 h dark cycle. All three subgenomes of BSMV strain ND18 were carried in the pBluescript plasmids pBS-BSMVα, pBS-BSMVβ and pBS-BSMVγ obtained from Large Scale Biology Corp. Vacaville, California, USA. The silencing construct pγb:PDS-as was obtained from Stéphane Bieri. The fluorescence control pENTR-(ubi)-eYFP was provided by Simone Oberhänsli and the expression plasmid pMDC32 was obtained from Mark Curtis. The *Arabidopsis* tonoplast-localized TT12-YFP construct (MARINOVA *et al.* 2007) used as positive fluorescence control was a gift from Hanne Grob.

Table 8: Plasmids used for expression in agroinfiltrated *N. benthamiana*

Entry clone	**destination clone**	**description**
pCJR43[1]	pMDC32-AVR_{K1}	AVR_{K1} with stop codon
pCJR44[1]	pMDC32-AVR_{A10}	AVR_{A10} with stop codon
pENTR4-AVR_{A10}-*myc*	pMDC32-AVR_{A10}-*myc*	encoded C-terminal myc tag
pENTR4-*myc*-AVR_{A10}	pMDC32-*myc*-AVR_{A10}	encoded N-terminal myc tag
pENTR4-AVR_{K1}-*myc*	pMDC32-AVR_{K1}-*myc*	encoded C-terminal myc tag
pENTR4-*myc*-AVR_{K1}	pMDC32-*myc*-AVR_{K1}	encoded N-terminal myc tag
pENTR4-*Mla10*-(c)	n.d.	n.d.
pENTR-(ubi)-*Mla10*-(c)	pMDC32-*Mla10*	*Mla10* cDNA with stop codon
pENTR4-*Mla10-HA*	pMDC32-*Mla10-HA*	encoded C-terminal HA tag
pENTR4-*Mla10-YFP*	pMDC32-*Mla10-YFP*	encoded C-terminal YFP fusion
pENTR-*GUS*[2]	pMDC32-*GUS*	GUS control
n.d.	pGWB-*Bs3*[3]	*Bs3* R-gene, HR control
n.d.	pGWB-*AvrBs3*[3]	*AvrBs3* from *X. campestris pv. vesicatoria*, HR control

[1](RIDOUT *et al.* 2006), [2] Invitrogen, [3](ROMER *et al.* 2007), n.d. = not defined

4.3.2. Cloning of different BSMV constructs

4.3.2.1. Amplification of AVRA10, AVRK1 and YFP

AVR$_{A10}$ was amplified by the primer pair sse022/sse023 (see chapter 6.8) using pCJR44 as a template (RIDOUT *et al.* 2006), *AVR$_{K1}$* by the primers sse026/sse027 using pCJR43 as a template (RIDOUT *et al.* 2006), and YFP with the primer pair sse024/sse025 using as a template pENTR-(ubi)-eYFP. PCR amplifications were carried out using the *Pfu*Ultra high-fidelity DNA polymerase (Stratagene) following the manufacturer's manual with inclusion of 2.5% DMSO. All PCR reactions were performed in a PTC-200 thermocycler (Bio-Rad). The amplification conditions to obtain the PCR products were 2 min at 94°C, followed by 32 cycles of 20 sec at 94°C, 30 sec at 55°C, 60 sec at 72°C, and then a final extension for 5min at 72°C. Amplification products were purified using the GenElute^{IM} Gel Extraction Kit (Sigma-Aldrich) and cloned in the modified pBS-BSMVβ or pBS-BSMVγ plasmids described below and listed in Table 7. Amplification products and the vector were digested with the restriction enzymes *Nhe*I and *Sac*II (New England Biolabs). All PCR-generated clones were sequenced using Big Dye Terminator V3.1 following the manufacturer's protocol on an ABI3730 automated sequencer (Applied Biosystems). As a positive control for fluorescence, plasmid pENTR-(ubi)-eYFP was transiently overexpressed following the description under chapter 2.4. After 48 h, the leaves were investigated for YFP fluorescence.

4.3.2.2. Constructs with replacement of the viral coat protein

A cloning cassette was introduced by creating three different replacement constructs of the viral coat protein β-a (Table 7). These constructs were designed by modification of plasmid pBS-BSMVβ (chapter 4.3.1.). A cloning cassette carrying the sites recognized by the restriction enzymes *Nhe*I and *Sac*II was designed. For all three deletion constructs, the 536bp long fragment amplified by the primers sse030/sse028 from pBS-BSMVβ was used. For all amplification fragments, the same PCR conditions were used as in chapter 4.3.2.1, except that 5% glycerol were added to the reaction volumes instead of 2.5% DMSO and with an annealing temperature of 50°C instead of 55°C. The plasmid p:β-Del1 contained a deletion of half of the coat protein designed by amplification of a 532 bp long PCR product by the primer pair sse029/sse031 using pBS-BSMVβ as a template. Both amplification products were purified using the GenElute^{IM} Gel Extraction Kit. For the overlap extension PCR approach, the amplifications were carried out using the *Pfu*Ultra high-fidelity DNA polymerase following the manufacturer's manual to obtain a 1044 bp long fragment. As template, 200ng

of each of the 532 bp and the 536 bp long fragments were sufficient. The following amplification conditions were used: 2 min at 94°C, followed by 6 cycles of 20 sec at 94°C, 40 sec at 50°C, 10 min at 68°C. After the 6 cycles, 2 µl sse030 (10 µM) and 2 µl sse031 (10 µM) were added to the PCR reaction, followed by a second amplification step: 2 min at 94°C, followed by 34 cycles of 20 sec at 94°C, 30 sec at 55°C, 2 min at 72°C and then a final extension for 5 min at 72°C. pBS-BSMVβ and the generated 1044 bp long fragment were digested using the restriction enzymes *Nae*I and *Nco*I (New England Biolabs) and ligated. The same strategy was used to construct plasmid p:β-Del2 (truncated version of the coat protein as described in HOLZBERG *et al.* 2002) and p:β-Del3 (deletion of the coat protein). For p:β-Del2, a 288 bp long gene product was amplified by the primers sse032/sse031 to generate an OE-PCR product with the 536 bp fragment generated with sse030/sse028 to obtain the 800 bp long gene product that was digested with the restriction enzymes *Nae*I and *Nco*I. The 749 bp long fragment was produced by a third OE-PCR with the 536 bp long sse030/sse028 product and the 237 bp long amplification product generated by the primer pair sse033/sse031.

4.3.2.3. Constructs with a modified γ-b protein

The insertion of a cloning cassette to create N-terminal, C-terminal fusion constructs with the γ-b protein including a complete replacement construct of the γ-b protein were designed by modifications of pBS-BSMVγ (Table 7 and chapter 4.3.1.). All PCR reactions were performed as described above. Three different cloning cassettes carrying the sites recognized by the restriction enzymes *Nhe*I and *Sac*II were designed. The N-terminal cloning cassette pγ:N was obtained by the amplification of a 1339 bp long fragment using the primer pair sbi216/sse042 and pBS-BSMVγ as template. To obtain pγ:N, both the vector and the amplification products were digested with *Kpn*I and *Bgl*II (New England Biolabs) and ligated. The C-terminal cloning cassette pγ:C was obtained by OE-PCR. The first 585 bp long PCR fragment was amplified by the primer pair sbi245/se044, the second 322 bp long fragment by the primers sse043/revl using pBS-BSMVγ as a template for both PCR amplifications. After the first six cycles of the OE-PCR, the primers sbi245 and revl were added to obtain the 883 bp long amplification product. pBS-BSMVγ and the 883 bp long amplified fragment were digested by the restriction enzymes *Nde*I and *Kpn*I and ligated to yield pγ:C. The third designed construct pγ:R involved a complete replacement of the γ-b sequence. A 1300 bp long fragment was amplified by the primer pair sbi216/sse045. The primer pair sse043 and revl were used to generate the second fragment. The two fragments were used to perform an OE-

PCR to obtain a 1599 bp long PCR amplification product. The restriction enzymes *Bgl*II and *Nde*I were used to digest the 1599 bp long product and pBS-BSMVγ to obtain the pγ:R.

4.3.3. Cloning of Gateway-compatible plasmids

4.3.3.1. pENTR4 plasmids generated by cloning with restriction enzymes

First, all constructs were cloned into pENTR4 (Invitrogen) by using restriction enzymes and then subcloned into the expression vector pMDC32 (obtained from Mark Curtis) by the Gateway technology (Inivtrogen), which is explained in detail under chapter 3.4.3. To obtain pENTR4-*Mla*10-HA with a C-terminal HA tag, a 2936 bp long amplification product was obtained with the primer pair sse091 and sse100. PCR amplifications were carried out using the *Pfu*Ultra high-fidelity DNA polymerase as described under 4.3.2.1. The PCR conditions were 2 min at 94°C, followed by 32 cycles of 20 sec at 94°C, 40 sec at 50°C, 10 min at 68°C, and then a final extension for 7 min at 68°C. The C-terminal YFP fusion construct pENTR4-Mla10-YFP was obtained by OE-PCR mentioned under chapter 3.4.1. The first two amplification products were generated by primers sse91/sse99 (2902 bp) and sse97/sse98 (2254 bp) to obtain a 3626 bp long fragment. Both, the plasmid pENTR4 and the amplification products of *Mla10* were digested with the restriction enzymes *BamH*I and *Not*I (New England Biolabs) and ligated. For the C-terminal myc-tagged pENTR4-*myc*-AVR_{A10}, the primer pair sse102/sse103 was used to amplify a 913 bp long PCR fragment. For the N-terminal myc-tagged pENTR4-AVR_{A10}-*myc*, the primer pair sse104/sse105 generated a fragment of the same size. A 586 bp long amplification product was obtained with the primers sse106/sse107 to clone pENTR4-AVR_{K1}-*myc* and sse108/sse109 for pENTR4-*myc*-AVR_{K1}. All PCR fragments were obtained under the same conditions used above, except that the final extension was 5 min at 68°C. Both the fragments and pENTR4 were digested with the restriction enzymes *Nco*I and *Not*I and ligated.

4.3.3.2. Sub cloning into the destination vector pMDC32 using the Gateway system

pENTR4 plasmids containing inserts flanked by *attL* sites were recombined into the destination vector pMDC32 using the Gateway system (Invitrogen) following the manual's instructions and explained in detail under chapter 3.4.3. All entry clones and expression constructs are listed in Table 8. For the construct pMDC32-*Mla10*, the entry clone was modified by the insertion of an ubiquitin promoter (see chapter 2). After recombination to pMDC32, the plasmid was digested with the restriction enzyme *Asc*I to remove the ubiquitin

promoter. After purification by the GenElute^{IM} Gel Extraction Kit, the 13,562 bp fragment was self-ligated.

4.3.4. Isolation of *Mla9* from cDNA

The isolation of the *Mla*9 sequence was performed as described in 2.4.2. RNA was isolated from the two near-isogenic barley lines Pallas P07 (Monte Cristo (*Mla9*), NGB 4937) and Pallas P17 (Monte Cristo (*Mlk*), NGB4948). The 3197 bp long sequence of *Mla9* was amplified from both cultivars by the primer pair sbi184/sbi299 and partially sequenced with the two primers unil and revl.

4.3.5. BSMV-induced gene silencing

Gene silencing mediated by Barley Stripe Mosaic Virus (BSMV) was performed as described in SHEN *et al.* (2007). 10 µg of the pBS-BMSVα and of the pBS-BSMVγ plasmids were linearized with 200U of *Mlu*I (New England Biolabs), 5 µg pBS-BSMVβ with 200 U of *Spe*I (NEB) and 5 µg pBS-BSMVγ-b-x with 200 U of *BssH*II (Roche, #1168851001). The DNA was precipitated with 0.1 volumes 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol. The linearized DNA was dissolved in TE buffer to a concentration of 500 ng/µl. *In vitro* transcription of the three BMSV subgenomes was performed using the T7 mMessage mMaschine Kit (Ambion, Catalog #1344) by following the instructor's manual (see Appendix 6.2 for an example protocol). The mixture containing 500 ng/µl of linearized plasmid was incubated at 37°C for one hour. 1 µl of each reaction was analysed on a 1% agarose gel. For one plant, 1 µl of each of the three viral transcripts were mixed, frozen in liquid nitrogen and stored at -80°C until further use. For plant inoculation, the generated viral transcripts were mixed with 22 µl FES inoculation buffer per plant (for details see chapter 6.5.). To inoculate the plants, 25 µl of the FES buffer mixture were put on finger (index and thumb with latex gloves) to inoculate the primary leaf of one or two five days old seedlings by holding the plant carefully with the other hand on the base to avoid leave rupture. The leaf was gently inoculated by gliding several times toward the tip in a single motion until a squeaking sound occurred. After inoculation, a cover was watered to protect the plants against dryness for 12 h. Five days old barley seedlings were used for inoculation with the viral transcripts. After two weeks, the plants were analyzed for BSMV infection symptoms.

4.3.6. Infiltration of *N. benthamiana*

For overexpression in *N. benthamiana,* constructs were first transformed into *A. tumefaciens* as described in chapter 3.4.5. Agrobacterium-mediated infiltration of *N. benthamiana* was performed as described in detail under chapter 3.4.7. The pepper resistance gene *Bs3* and its cognate effector gene *AvrBs3* (ROMER *et al.* 2007) were co-expressed in *N. benthamiana* as a positive control. After three days, a fully developed HR visible as necrosis in the agroinfiltrated area developed.

5. Outlook

5.1 Investigation of the complex Mla locus by the "next generation" sequencing technologies

To study the previously described diversity at the resistance gene locus *Mla* in barley, we have chosen a PCR-based approach (for details see chapter 2). The investigation of 36 barley cultivars resulted in the isolation of 23 new candidate MLA cDNAs. Our isolation strategy was based on the knowledge of six previously isolated MLA cDNAs, which showed a highly polymorphic 3'end for all *Mla* genes. The new sequences revealed that all 29 validated and candidate MLA cDNAs are highly diverse in the encoded LRR domain. We chose cDNA as a template, because it has the advantage to amplify only alleles that are expressed and because attempts to amplify *Mla* from genomic DNA failed for unknown reasons. To tackle the complexity of a diverse *Mla* 3'end, we performed a 3'RACE to obtain sequence information up- and downstream of the stop codon. In retrospect, we think that our strategy was in fact the best way to characterise the *Mla* genes of the 36 barley cultivars. Nevertheless, we assume that our PCR-based approach is not efficient enough for screening a large set of barley lines, particularly wild barley, to investigate the *Mla* locus in more detail.

Thus, it may be more effective to obtain further information about the complex *Mla* locus by directly sequencing the individual genomes of all barley cultivars of interest by the new and promising "next generation" sequencing technologies. At the moment, sequencing of individual genomes would be highly expensive either by the classical Sanger sequencing (the chain termination method) or by using the new sequencing methods. Nevertheless, it is expected that in the near future the sequencing of individual genomes from one species will become cost effective by using the new technologies (HALL 2007). Additionally, these methods will be much faster than the classical Sanger sequencing, as they rely on amplified single DNA fragments instead of cloned fragments in plasmids and thus, can produce much more sequences (GUPTA 2008; WOLD and MYERS 2008). Thus, re-sequencing several individual genomes of one species like barley is proposed to be a new and promising approach to tackle many complex issues in a fast and elegant way.

At the moment, bioinformatic tools have to be developed to deal with the problems of the new sequencing methods like the short read lengths, higher error-rates and the difficulty of

managing massive amounts of data (GUPTA 2008). For example, in the 454 technology (Roche Applied Sciences) the read length achieved at the moment approaches 300 bp, which is still shorter than the 700-900bp achieved by Sanger sequencing (MOROZOVA and MARRA 2008).

The sequencing of individual barley cultivar genomes and the comparison of the different *Mla* loci will show us how many *Mla* homologues are present at each *Mla* locus. Such a study will reveal how many barley cultivars harbour more than one *Mla* gene and if *Mla* varieties with a novel 5'end are widely distributed (for details see chapter 3). Many new insights could be obtained by exploring the diversity at the *Mla* locus in *Hordeum spontaneum*, the closest wild relative of cultivated barley, as this gene pool was widely used by breeders to obtain new powdery mildew resistances (JAHOOR and FISCHBECK 1987; JAHOOR and FISCHBECK 1993). If the geographical position of each wild barley plant is known, population genetic studies can be performed concerning the frequencies of each studied *Mla* gene.

5.2 Investigation and characterization of novel CC domains

It would be interesting to test the physical interaction between $TmMLA_{1\text{-}46}$ with HvWRKY1, as shown in chapter 3, by an additional method like the pull-down assay or bimolecular fluorescent complementation assay (BiFC). BiFC is a technique with the advantage of showing protein-protein interaction in living cells and should be handled more easily than FRET or FLIM-FRET assays (OHAD *et al.* 2007). For BiFC, YFP is split into non-overlapping N-terminal (SPYNE) und C-terminal (SPYCE) fragments, which are tagged to one of the two interaction partners (MESSINESE *et al.* 2007).

Neither MLA18-$1_{1\text{-}46}$ nor MLA25-$1_{1\text{-}46}$ showed an interaction with the two transcription factors HvWRKY1 and HvWRKY2 in the yeast two-hybrid tests (chapter 3). This result contrasts with the intermediate resistance responses observed for the two domain swap experiments with MLA1, where the first 46 residues were derived either from MLA18-1 or MLA25-1 (chapter 3). Thus, it is possible that a protein other than HvWRKY1/2 interacts with these two novel N-termini. A recent study identified 45 different WRKY genes in barley and grouped them according to sequence identity into three different classes (MANGELSEN *et al.* 2008). The group with HvWRKY1 and HvWRKY2 includes two additional barley WRKY proteins called HvWRKY3 and HvWRKY23 (MANGELSEN *et al.* 2008). These two

transcription factors are potential regulators of defence mechanisms against pathogens like powdery mildew. Thus, it could be examined if HvWRKY3 or HvWRKY23 also interact with the N-terminus of MLA10, and whether they interact with one of the three novel N-termini of MLA18-1, MLA25-1 or TmMLA1.

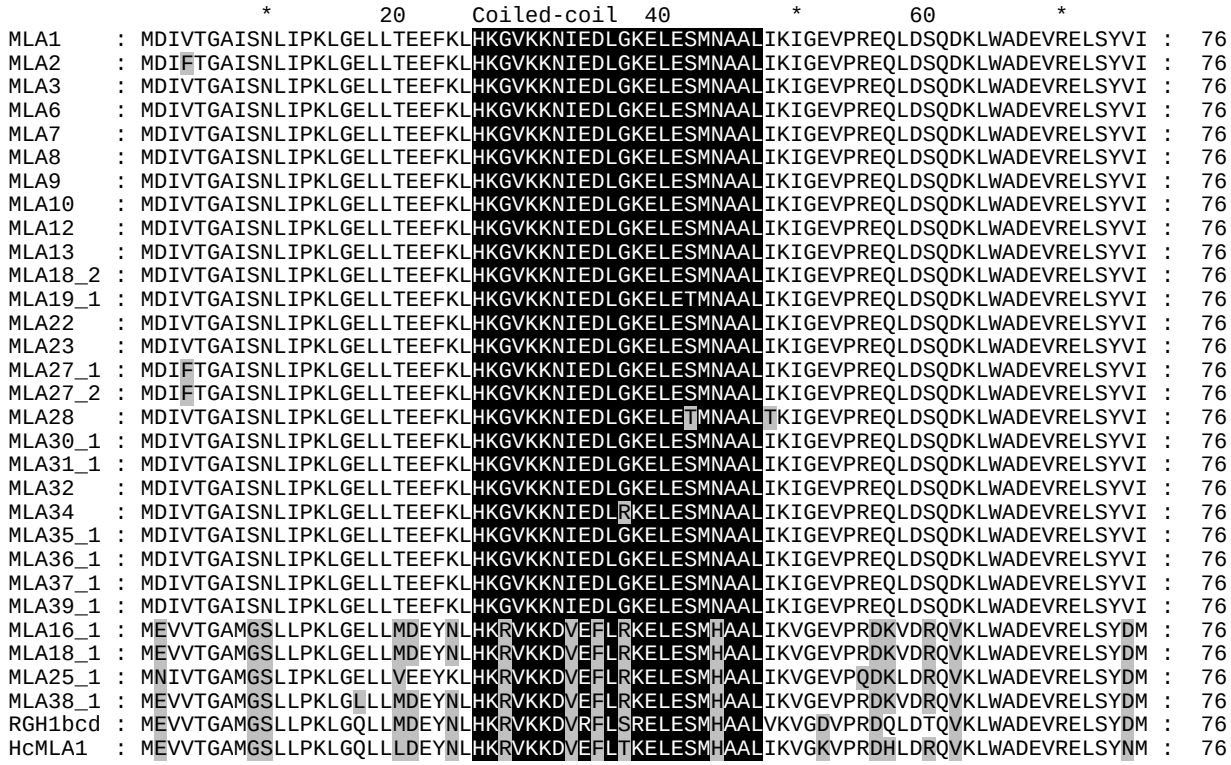
It is not known if MLA18-1 and MLA25-1 are involved in recognition specificity or in other unknown regulatory mechanisms. It is possible that an unknown interaction partner different from the WRKY transcription factors interact with the novel N-termini. Thus, a promising strategy could be a yeast two-hybrid screen with the first 46 residues of MLA18-1 or of MLA25-1 instead of testing them only against HvWRKY3 and HvWRKY23. Gene expression profiles of different HvWRKY genes revealed that most members show increased expression at different time points after powdery mildew inoculation (MANGELSEN *et al.* 2008), which could also be the case for other potential interaction proteins. Thus, it would be essential for the yeast two-hybrid screen to consider several cDNA libraries constructed from different time points after inoculation to avoid missing the interaction partner of MLA18-1 or MLA25-1.

Finally, purification of the C-termini of HvWRKY1 and HvWRKY2 and $MLA10_{1\text{-}46}$ would be important to solve the structure of the protein-protein complex either by X-ray or NMR techniques. The obtained information would shed further light on the residues involved in the interaction between MLA10 and HvWRKY1 or HvWRKY2. Additionally, it could answer the question why the novel CC domains of MLA18-1 and MLA25-1 do not interact with these two transcription factors in the yeast two-hybrid assay.

6. Appendix

6.1 Alignment of all 28 MLA sequences at the amino acid level

Black: motifs conserved among the different MLA proteins; orange: the 34 sites of positive selection for a set of 25 MLA sequences (see Figure 7); blue: first amino acid of a new domain. The CC domain is from residue 1 to 160 (for MLA1) including the predicted coiled-coil (22 to 47). The NB domain (160-549) consists of several conserved motifs (VAN OOIJEN *et al.* 2008). Grey: polymorphic amino acids in the CC and NB domain. The LRR domain (from 550 to 958 for MLA1) is illustrated with the 15 LxxLxLxx motifs predicted to form the solvent-exposed β-strand (black: L positions, grey: x positions; KAJAVA *et al.* 1995). MLA38-1 is truncated due to a stop codon at position 233 and RGH1bcd is only 712 amino acids long.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MLA3 : MDIVTGAISNLIPKLGELLTEEFKLHKGVKKNIEDLGKELESMNAALIKIGEVPREQLDSQDKLWADEVRELSYVI : 76
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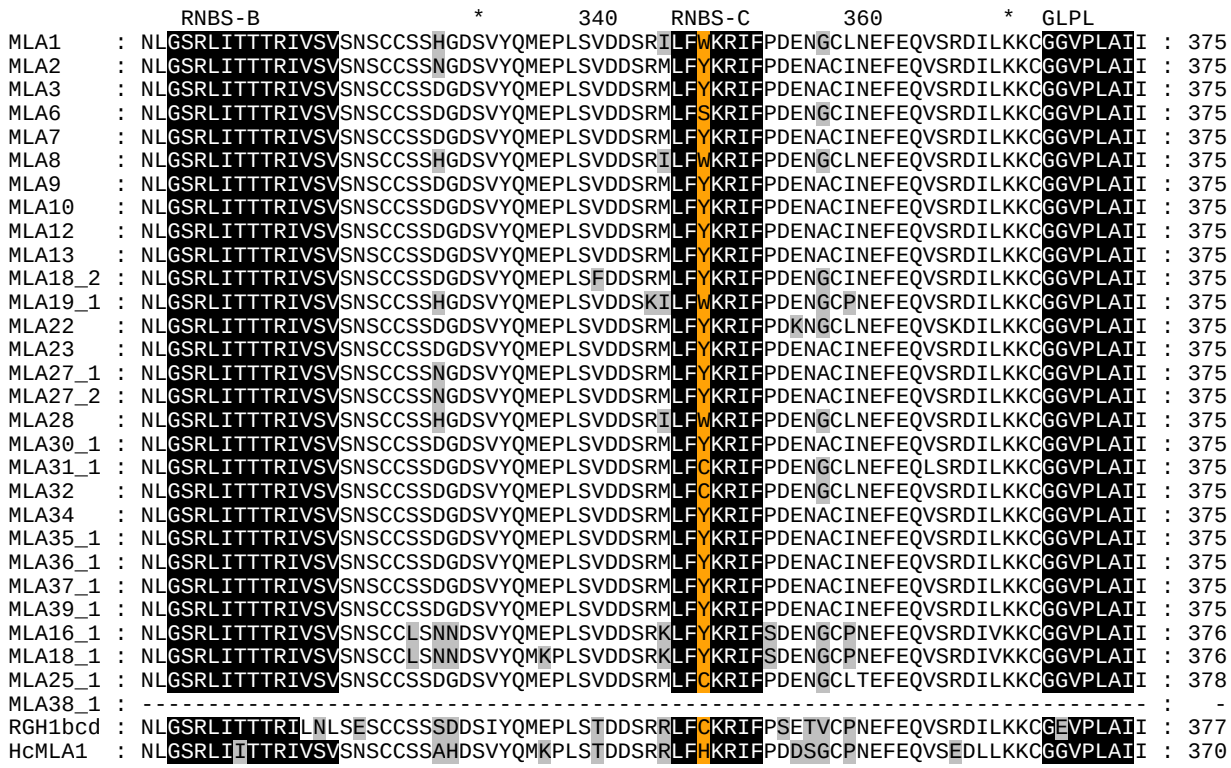
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MLA25_1 : NLGSRLITTTRIVSVSNSCCSSDGDSVYQMEPLSVDDSRMLFCKRIFPDENGCLTEFEQVSRDILKKCGGVPLAII : 378
MLA38_1 : ---------- : -
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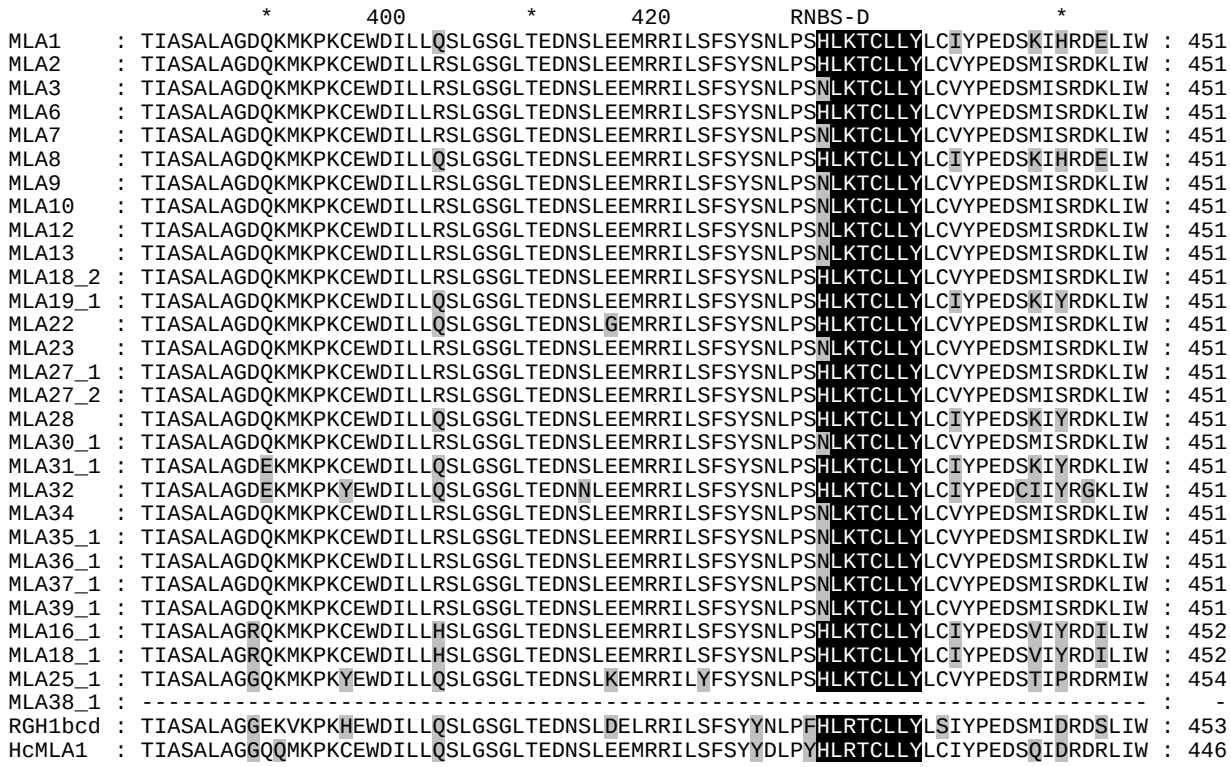
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MLA2 : TIASALAGDQKMKPKCEWDILLRSLGSGLTEDNSLEEMRRILSFSYSNLPSHLKTCLLYLCVYPEDSMISRDKLIW : 451
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MLA18_1 : TIASALAGRQKMKPKCEWDILLHSLGSGLTEDNSLEEMRRILSFSYSNLPSHLKTCLLYLCIYPEDSVIYRDILIW : 452
MLA25_1 : TIASALAGGQKMKPKYEWDILLQSLGSGLTEDNSLKEMRRILYFSYSNLPSHLKTCLLYLCVYPEDSTIPRDRMIW : 454
MLA38_1 : ---------- : -
RGH1bcd : TIASALAGGEKVKPKHEWDILLQSLGSGLTEDNSLDELRRILSFSYYNLPFHLRTCLLYLSIYPEDSMIDRDSLIW : 453
HcMLA1 : TIASALAGGQQMKPKCEWDILLQSLGSGLTEDNSLEEMRRILSFSYYDLPYHLRTCLLYLCIYPEDSQIDRDRLIW : 446

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            460         *       480         *       500    MHD          520         *
MLA1     : KWVAEGFVHHENQGNSLYLLGLNYFNQLINRSMIQPIYGFNDEVYVCRVHDMVLDLICNLSREAKFVNLLDGSGNS : 527
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MLA31_1  : KWVAEGFVHHQNQGSSLFLLGLNYFNQLINRSMIQPIYGITDKVYACRVHDMVLDLICNLSHEAKFVNLLDGTRN- : 526
MLA32    : KWVAEGFVHHENQGNSLYLLGLNYFNQLINRSMIQPIYNYSGEAYACHVHDMVLDLICNLSHEAKFVNLLDDTGNS : 527
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MLA25_1  : KWMAEGFVHHGNQGTSLFLLGLNYFNELVNRSMIQPIYGTTGEVYACRVHDMVLDLICNLSYEAKFVNLLDGTGNS : 530
MLA38_1  : ------------------------------------------------------------------------------ :   -
RGH1bcd  : KWVAEGFVHHGNQGTSLFLLGLNFFNQLINRSLIQPIYSFSGDVHACRVHDMVLDLICNLSHEAKFVNLLDGTGNS : 529
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MLA13    : MSSQSNCRRLSLQKRNEDHQARPFTDIKSMSRVRSITIFPSAIEVMPSLSRFDVLRVLDLSRCNLGENSSMQLNLK : 603
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MLA22    : MSSQSNYRRLSIQNRNEDHQAKPLTDIKSMSRVRSITIFPPSIKVMPSLSRFEVLRVLDLSKCNLGENSSLQLNLK : 603
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MLA38_1  : ------------------------------------------------------------------------------ :   -
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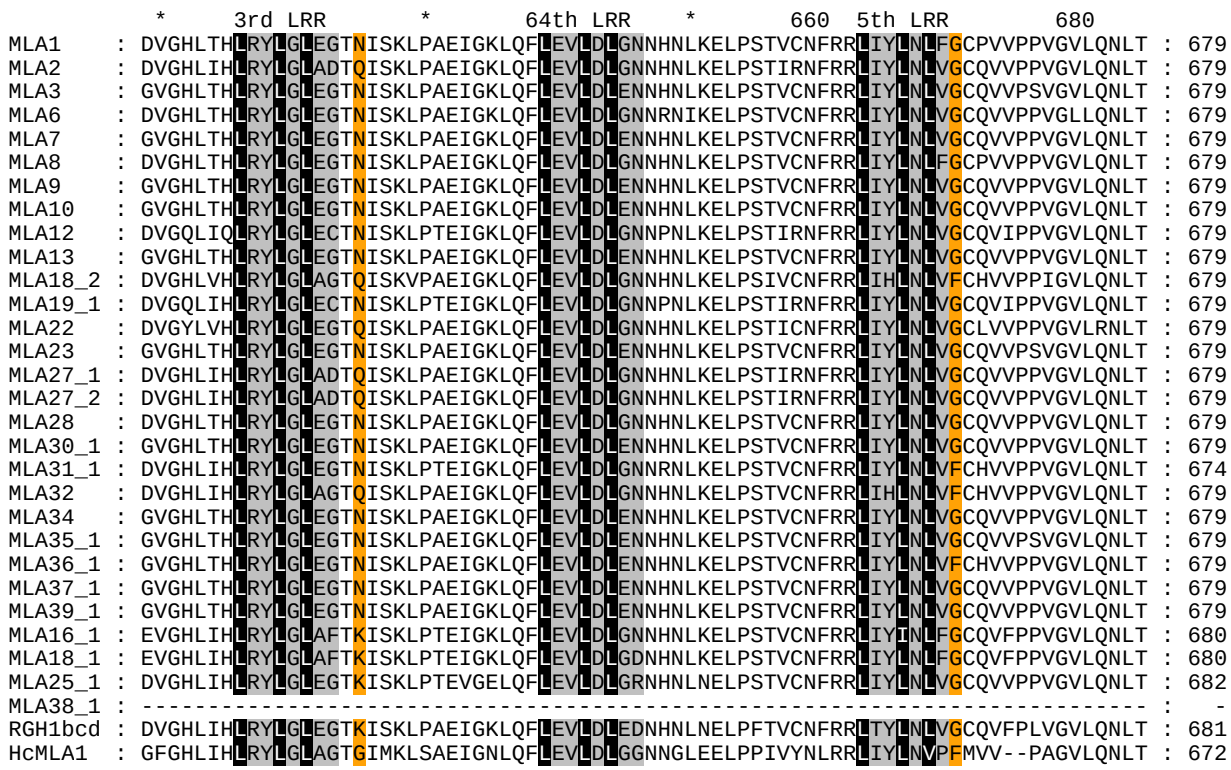
* 3rd LRR * 64th LRR * 660 5th LRR 680
MLA1 : DVGHLTHLRYLGLEGTNISKLPAEIGKLQFLEVLDLGNNHNLKELPSTVCNFRRLIYLNLFGCPVVPPVGVLQNLT : 679
MLA2 : DVGHLIHLRYLGLADTQISKLPAEIGKLQFLEVLDLGNNHNLKELPSTIRNFRRLIYLNLVGCQVVPPVGVLQNLT : 679
MLA3 : GVGHLTHLRYLGLEGTNISKLPAEIGKLQFLEVLDLENNHNLKELPSTVCNFRRLIYLNLVGCQVVPSVGVLQNLT : 679
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MLA16_1 : EVGHLIHLRYLGLAFTKISKLPTEIGKLQFLEVLDLGNNHNLKELPSTVCNFRRLIYINLFGCQVFPPVGVLQNLT : 680
MLA18_1 : EVGHLIHLRYLGLAFTKISKLPTEIGKLQFLEVLDLGDNHNLKELPSTVCNFRRLIYLNLFGCQVFPPVGVLQNLT : 680
MLA25_1 : DVGHLIHLRYLGLEGTKISKLPTEVGELQFLEVLDLGRNHNLNELPSTVCNFRRLIYLNLVGCQVVPPVGVLQNLT : 682
MLA38_1 : ---------- : -
RGH1bcd : DVGHLIHLRYLGLEGTKISKLPAEIGKLQFLEVLDLEDNHNLNELPFTVCNFRRLTYLNLVGCQVFPLVGVLQNLT : 681
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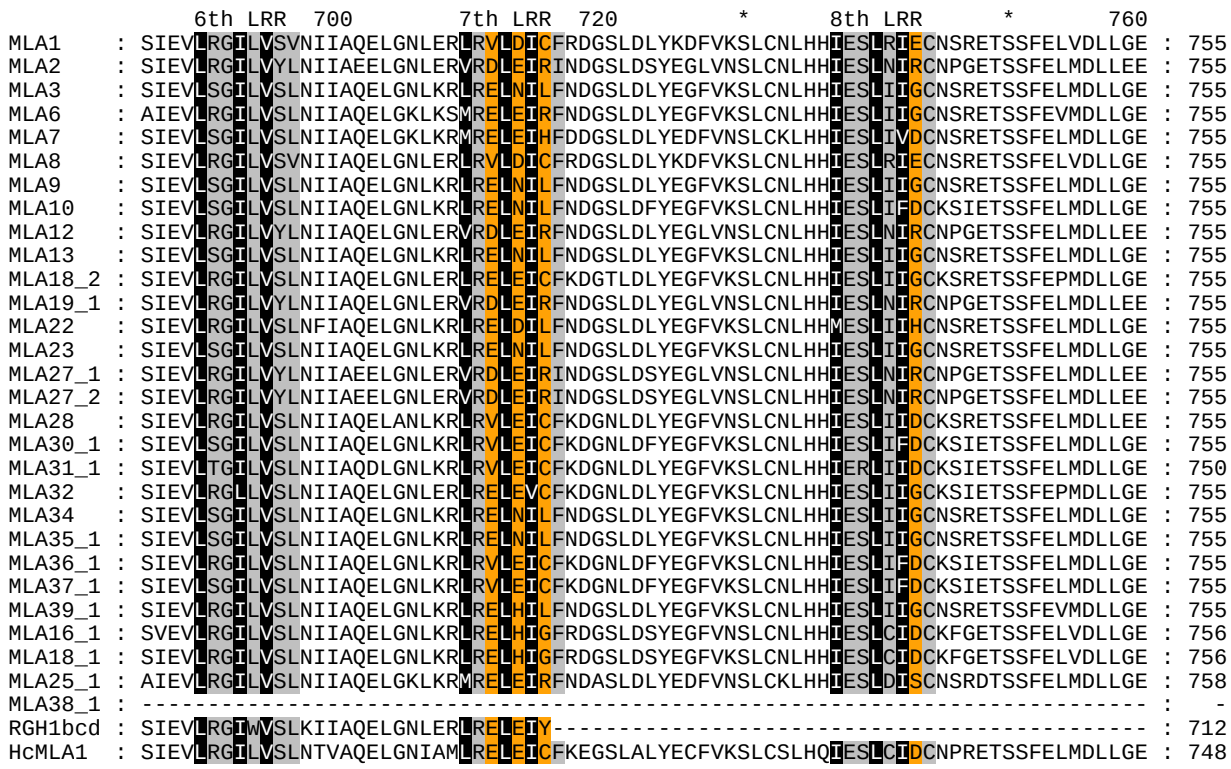
6th LRR 700 7th LRR 720 * 8th LRR * 760
MLA1 : SIEVLRGILVSVNIIAQELGNLERLRVLDICFRDGSLDLYKDFVKSLCNLHHIESLRIECNSRETSSFELVDLLGE : 755
MLA2 : SIEVLRGILVYLNIIAEELGNLERVRDLEIRINDGSLDSYEGLVNSLCNLHHIESLNIRCNPGETSSFELMDLLEE : 755
MLA3 : SIEVLSGILVSLNIIAQELGNLKRLRELNILFNDGSLDLYEGFVKSLCNLHHIESLIIGCNSRETSSFELMDLLGE : 755
MLA6 : AIEVLRGILVSLNIIAQELGKLKSMRELEIRFNDGSLDLYEGFVKSLCNLHHIESLIIGCNSRETSSFEVMDLLGE : 755
MLA7 : SIEVLSGILVSLNIIAQELGKLKRMRELEIHFDDGSLDLYEDFVNSLCKLHHIESLIVDCNSRETSSFELMDLLGE : 755
MLA8 : SIEVLRGILVSVNIIAQELGNLERLRVLDICFRDGSLDLYKDFVKSLCNLHHIESLRIECNSRETSSFELVDLLGE : 755
MLA9 : SIEVLSGILVSLNIIAQELGNLKRLRELNILFNDGSLDLYEGFVKSLCNLHHIESLIIGCNSRETSSFELMDLLGE : 755
MLA10 : SIEVLSGILVSLNIIAQELGNLKRLRELNILFNDGSLDFYEGFVKSLCNLHHIESLIFDCKSIETSSFELMDLLGE : 755
MLA12 : SIEVLRGILVYLNIIAQELGNLERVRDLEIRFNDGSLDLYEGLVNSLCNLHHIESLNIRCNPGETSSFELMDLLEE : 755
MLA13 : SIEVLSGILVSLNIIAQELGNLKRLRELNILFNDGSLDLYEGFVKSLCNLHHIESLIIGCNSRETSSFELMDLLGE : 755
MLA18_2 : SIEVLRGILVSLNIIAQELGNLERLRELEICFKDGTLDLYEGFVKSLCNLHHIESLIIGCKSRETSSFEPMDLLGE : 755
MLA19_1 : SIEVLRGILVYLNIIAQELGNLERVRDLEIRFNDGSLDLYEGLVNSLCNLHHIESLNIRCNPGETSSFELMDLLEE : 755
MLA22 : SIEVLRGILVSLNFIAQELGNLKRLRELDILFNDGSLDLYEGFVKSLCNLHHMESLIIHCNSRETSSFELMDLLGE : 755
MLA23 : SIEVLSGILVSLNIIAQELGNLKRLRELNILFNDGSLDLYEGFVKSLCNLHHIESLIIGCNSRETSSFELMDLLGE : 755
MLA27_1 : SIEVLRGILVYLNIIAEELGNLERVRDLEIRINDGSLDSYEGLVNSLCNLHHIESLNIRCNPGETSSFELMDLLEE : 755
MLA27_2 : SIEVLRGILVYLNIIAEELGNLERVRDLEIRINDGSLDSYEGLVNSLCNLHHIESLNIRCNPGETSSFELMDLLEE : 755
MLA28 : SIEVLRGILVSLNIIAQELANLKRLRVLEICFKDGNLDLYEGFVNSLCNLHHIESLIIDCKSRETSSFELMDLLEE : 755
MLA30_1 : SIEVLSGILVSLNIIAQELGNLKRLRVLEICFKDGNLDFYEGFVKSLCNLHHIESLIFDCKSIETSSFELMDLLGE : 755
MLA31_1 : SIEVLTGILVSLNIIAQDLGNLKRLRVLEICFKDGNLDLYEGFVKSLCNLHHIERLIIDCKSIETSSFELMDLLGE : 750
MLA32 : SIEVLRGLLVSLNIIAQELGNLERLRELEVCFKDGNLDLYEGFVKSLCNLHHIESLIIGCKSIETSSFEPMDLLGE : 755
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MLA35_1 : SIEVLSGILVSLNIIAQELGNLKRLRELNILFNDGSLDLYEGFVKSLCNLHHIESLIIGCNSRETSSFELMDLLGE : 755
MLA36_1 : SIEVLRGILVSLNIIAQELGNLKRLRVLEICFKDGNLDFYEGFVKSLCNLHHIESLIFDCKSIETSSFELMDLLGE : 755
MLA37_1 : SIEVLSGILVSLNIIAQELGNLKRLRVLEICFKDGNLDFYEGFVKSLCNLHHIESLIFDCKSIETSSFELMDLLGE : 755
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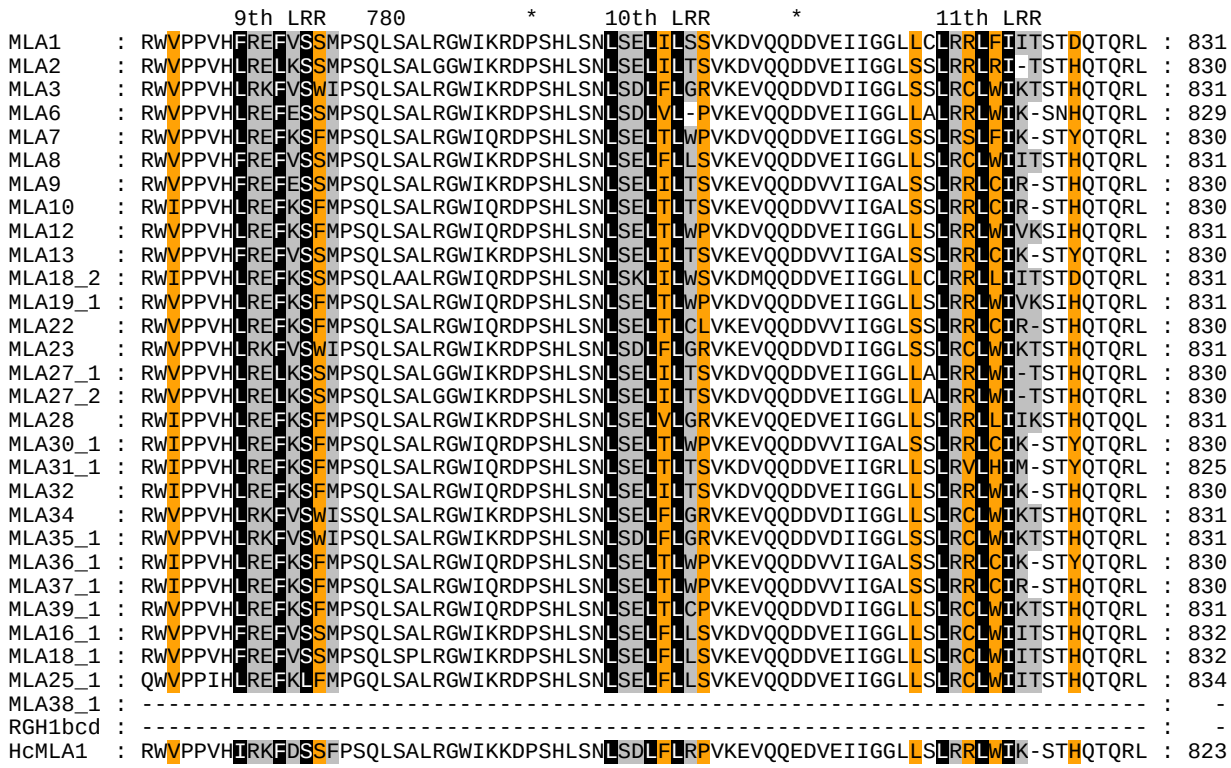
MLA18_1 : SIEVLRGILVSLNIIAQELGNLKRLRELHIGFRDGSLDSYEGFVKSLCNLHHIESLCIDCKFGETSSFELVDLLGE : 756
MLA25_1 : AIEVLRGILVSLNIIAQELGKLKRMRELEIRFNDASLDLYEDFVNSLCKLHHIESLDISCNSRDTSSFELMDLLGE : 758
MLA38_1 : ---------- : -
RGH1bcd : SIEVLRGIWVSLKIIAQELGNLERLRELEIY---------- : 712
HcMLA1 : SIEVLRGILVSLNTVAQELGNIAMLRELEICFKEGSLALYECFVKSLCSLHQIESLCIDCNPRETSSFELMDLLGE : 748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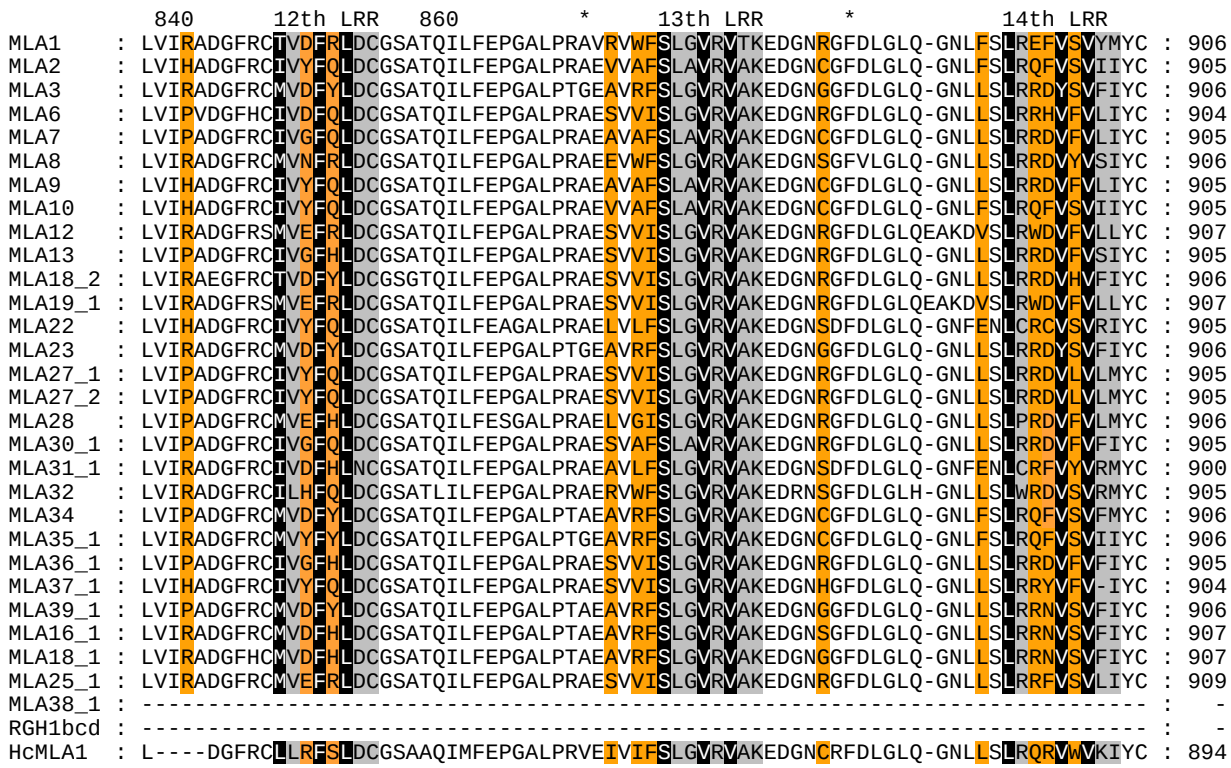
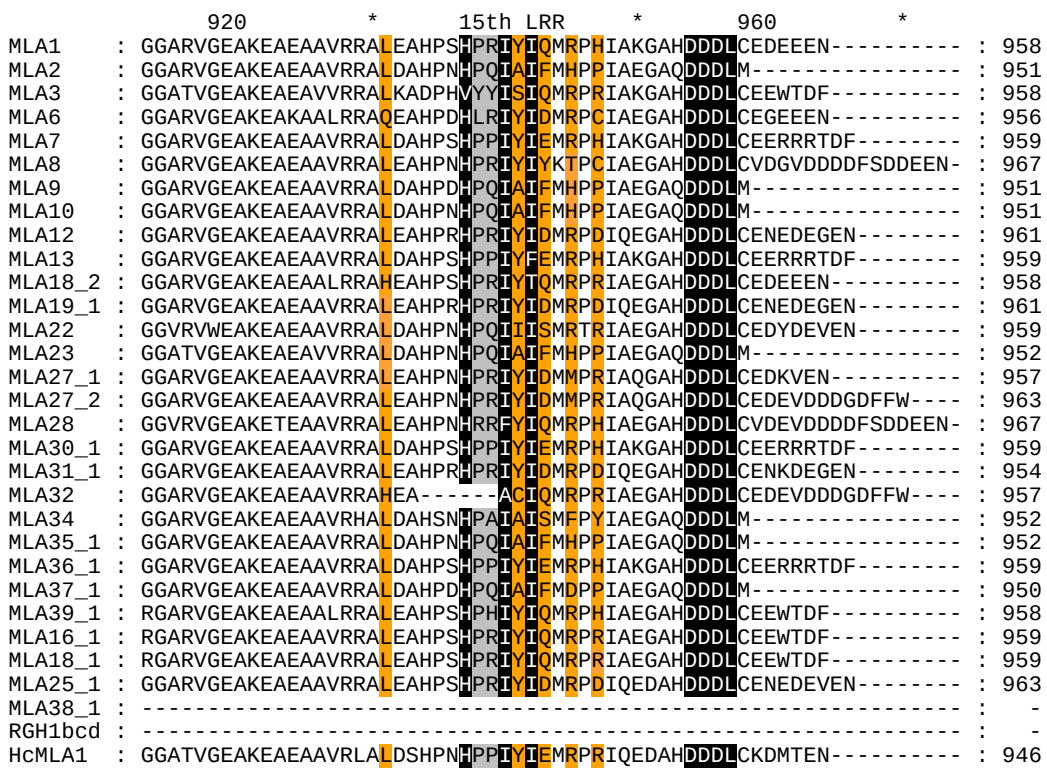
MLA2 : RWVPPVHLREFKSSMPSQLSALGGWIKRDPSHLSNLSELILTSVKDVQQDDVEIIGGLSSLRRLRI-TSTHQTQRL : 830
MLA3 : RWVPPVHLRKFVSWIPSQLSALRGWIKRDPSHLSNLSDLFLGRVKEVQQDDVDIIGGLSSLRCLWIKTSTHQTQRL : 831
MLA6 : RWVPPVHLREFESSMPSQLSALRGWIKRDPSHLSNLSDLVL-PVKEVQQDDVEIIGGLLALRRLWIK-SNHQTQRL : 829
MLA7 : RWVPPVHLREFKSFMPSQLSALRGWIQRDPSHLSNLSELTLWPVKDVQQDDVEIIGGLSSLRSLFIK-STYQTQRL : 830
MLA8 : RWVPPVHFREFVSSMPSQLSALRGWIKRDPSHLSNLSELFLLSVKEVQQDDVEIIGGLLSLRCLWIITSTHQTQRL : 831
MLA9 : RWVPPVHFREFESSMPSQLSALRGWIKRDPSHLSNLSELILTSVKEVQQDDVVIIGALSSLRRLCIR-STHQTQRL : 830
MLA10 : RWIPPVHLREFKSFMPSQLSALRGWIQRDPSHLSNLSELTLTSVKEVQQDDVVIIGALSSLRRLCIR-STHQTQRL : 830
MLA12 : RWVPPVHLREFKSFMPSQLSALRGWIQRDPSHLSNLSELTLWPVKDVQQDDVEIIGGLLSLRRLWIVKSIHQTQRL : 831
MLA13 : RWVPPVHFREFVSSMPSQLSALRGWIKRDPSHLSNLSELILTSVKEVQQDDVVIIGALSSLRRLCIK-STYQTQRL : 830
MLA18_2 : RWIPPVHLREFKSSMPSQLAALRGWIQRDPSHLSNLSKLILWSVKDMQQDDVEIIGGLLCLRRLLIITSTDQTQRL : 831
MLA19_1 : RWVPPVHLREFKSFMPSQLSALRGWIQRDPSHLSNLSELTLWPVKDVQQDDVEIIGGLLSLRRLWIVKSIHQTQRL : 831
MLA22 : RWVPPVHLREFKSFMPSQLSALRGWIQRDPSHLSNLSELTLCLVKEVQQDDVVIIGGLSSLRRLCIR-STHQTQRL : 830
MLA23 : RWVPPVHLRKFVSWIPSQLSALRGWIKRDPSHLSNLSDLFLGRVKEVQQDDVDIIGGLSSLRCLWIKTSTHQTQRL : 831
MLA27_1 : RWVPPVHLRELKSSMPSQLSALGGWIKRDPSHLSNLSELILTSVKDVQQDDVEIIGGLLALRRLWI-TSTHQTQRL : 830
MLA27_2 : RWVPPVHLRELKSSMPSQLSALGGWIKRDPSHLSNLSELILTSVKDVQQDDVEIIGGLLALRRLWI-TSTHQTQRL : 830
MLA28 : RWIPPVHLREFKSFMPSQLSALRGWIKRDPSHLSNLSELVLGRVKEVQQEDVEIIGGLLSLRRLLIIKSTHQTQQL : 831
MLA30_1 : RWIPPVHLREFKSFMPSQLSALRGWIQRDPSHLSNLSELTLWPVKEVQQDDVVIIGALSSLRRLCIK-STYQTQRL : 830
MLA31_1 : RWIPPVHLREFKSFMPSQLSALRGWIQRDPSHLSNLSELTLTSVKDVQQDDVEIIGRLLSLRVLHIM-STYQTQRL : 825
MLA32 : RWIPPVHLREFKSFMPSQLSALRGWIKRDPSHLSNLSELILTSVKEVQQDDVEIIGGLLSLRRLWIK-STHQTQRL : 830
MLA34 : RWVPPVHLRKFVSWISSQLSALRGWIKRDPSHLSNLSELFLGRVKEVQQDDVDIIGGLLSLRCLWIKTSTHQTQRL : 831
MLA35_1 : RWVPPVHLRKFVSWIPSQLSALRGWIKRDPSHLSNLSDLFLGRVKEVQQDDVDIIGGLSSLRCLWIKTSTHQTQRL : 831
MLA36_1 : RWIPPVHLREFKSFMPSQLSALRGWIQRDPSHLSNLSELTLWPVKEVQQDDVVIIGALSSLRRLCIK-STYQTQRL : 830
MLA37_1 : RWIPPVHLREFKSFMPSQLSALRGWIQRDPSHLSNLSELTLWPVKEVQQDDVVIIGALSSLRRLCIR-STHQTQRL : 830
MLA39_1 : RWVPPVHLREFKSFMPSQLSALRGWIQRDPSHLSNLSELTLCPVKEVQQDDVDIIGGLLSLRCLWIKTSTHQTQRL : 831
MLA16_1 : RWVPPVHFREFVSSMPSQLSALRGWIKRDPSHLSNLSELFLLSVKDVQQDDVEIIGGLLSLRCLWIITSTHQTQRL : 832
MLA18_1 : RWVPPVHFREFVSSMPSQLSPLRGWIKRDPSHLSNLSELFLLSVKEVQQDDVEIIGGLLSLRCLWIITSTHQTQRL : 832
MLA25_1 : QWVPPIHLREFKLFMPGQLSALRGWIKRDPSHLSNLSELFLLSVKEVQQDDVEIIGGLLSLRCLWIITSTHQTQRL : 834
MLA38_1 : ---------- : -
RGH1bcd : ---------- : -
HcMLA1 : RWVPPVHIRKFDSSFPSQLSALRGWIKKDPSHLSNLSDLFLRPVKEVQQEDVEIIGGLLSLRRLWIK-STHQTQRL : 823


840 12th LRR 860 * 13th LRR * 14th LRR
MLA1 : LVIRADGFRCTVDFRLDCGSATQILFEPGALPRAVRVWFSLGVRVTKEDGNRGFDLGLQ-GNLFSLREFVSVYMYC : 906
MLA2 : LVIHADGFRCIVYFQLDCGSATQILFEPGALPRAEVVAFSLAVRVAKEDGNCGFDLGLQ-GNLFSLRQFVSVIIYC : 905
MLA3 : LVIRADGFRCMVDFYLDCGSATQILFEPGALPTGEAVRFSLGVRVAKEDGNGGFDLGLQ-GNLLSLRRDYSVFIYC : 906
MLA6 : LVIPVDGFHCIVDFQLDCGSATQILFEPGALPRAESVVISLGVRVAKEDGNRGFDLGLQ-GNLLSLRRHVFVLIYC : 904
MLA7 : LVIPADGFRCIVGFQLDCGSATQILFEPGALPRAEAVAFSLAVRVAKEDGNCGFDLGLQ-GNLLSLRRDVFVLIYC : 905
MLA8 : LVIRADGFRCMVNFRLDCGSATQILFEPGALPRAEEVWFSLGVRVAKEDGNSGFVLGLQ-GNLLSLRRDVYVSIYC : 906
MLA9 : LVIHADGFRCIVYFQLDCGSATQILFEPGALPRAEAVAFSLAVRVAKEDGNCGFDLGLQ-GNLLSLRRDVFVLIYC : 905
MLA10 : LVIHADGFRCIVYFQLDCGSATQILFEPGALPRAEVVAFSLAVRVAKEDGNCGFDLGLQ-GNLFSLRQFVSVIIYC : 905
MLA12 : LVIRADGFRSMVEFRLDCGSATQILFEPGALPRAESVVISLGVRVAKEDGNRGFDLGLQEAKDVSLRWDVFVLLYC : 907
MLA13 : LVIPADGFRCIVGFHLDCGSATQILFEPGALPRAESVVISLGVRVAKEDGNRGFDLGLQ-GNLLSLRRDVFVSIYC : 905
MLA18_2 : LVIRAEGFRCTVDFYLDCGSGTQILFEPGALPRAESVVISLGVRVAKEDGNRGFDLGLQ-GNLLSLRRDVHVFIYC : 906
MLA19_1 : LVIRADGFRSMVEFRLDCGSATQILFEPGALPRAESVVISLGVRVAKEDGNRGFDLGLQEAKDVSLRWDVFVLLYC : 907
MLA22 : LVIHADGFRCIVYFQLDCGSATQILFEAGALPRAELVLFSLGVRVAKEDGNSDFDLGLQ-GNFENLCRCVSVRIYC : 905
MLA23 : LVIRADGFRCMVDFYLDCGSATQILFEPGALPTGEAVRFSLGVRVAKEDGNGGFDLGLQ-GNLLSLRRDYSVFIYC : 906
MLA27_1 : LVIPADGFRCIVYFQLDCGSATQILFEPGALPRAESVVISLGVRVAKEDGNRGFDLGLQ-GNLLSLRRDVLVLMYC : 905
MLA27_2 : LVIPADGFRCIVYFQLDCGSATQILFEPGALPRAESVVISLGVRVAKEDGNRGFDLGLQ-GNLLSLRRDVLVLMYC : 905
MLA28 : LVIPADGFRCMVEFHLDCGSATQILFESGALPRAELVGISLGVRVAKEDGNRGFDLGLQ-GNLLSLPRDVFVLMYC : 906
MLA30_1 : LVIPADGFRCIVGFQLDCGSATQILFEPGALPRAESVAFSLAVRVAKEDGNRGFDLGLQ-GNLLSLRRDVFVFIYC : 905
MLA31_1 : LVIRADGFRCIVDFHLNCGSATQILFEPGALPRAEAVLFSLGVRVAKEDGNSDFDLGLQ-GNFENLCRFVYVRMYC : 900
MLA32 : LVIRADGFRCILHFQLDCGSATLILFEPGALPRAERVWFSLGVRVAKEDRNSGFDLGLH-GNLLSLWRDVSVRMYC : 905
MLA34 : LVIPADGFRCMVDFYLDCGSATQILFEPGALPTAEAVRFSLGVRVAKEDGNCGFDLGLQ-GNLFSLRQFVSVFMYC : 906
MLA35_1 : LVIRADGFRCMVYFYLDCGSATQILFEPGALPTGEAVRFSLGVRVAKEDGNCGFDLGLQ-GNLFSLRQFVSVIIYC : 906
MLA36_1 : LVIPADGFRCIVGFHLDCGSATQILFEPGALPRAESVVISLGVRVAKEDGNRGFDLGLQ-GNLLSLRRDVFVFIYC : 905
MLA37_1 : LVIHADGFRCIVYFQLDCGSATQILFEPGALPRAESVVISLGVRVAKEDGNHGFDLGLQ-GNLLSLRRYVFV-IYC : 904
MLA39_1 : LVIPADGFRCMVDFYLDCGSATQILFEPGALPTAEAVRFSLGVRVAKEDGNGGFDLGLQ-GNLLSLRRNVSVFIYC : 906
MLA16_1 : LVIRADGFRCMVDFHLDCGSATQILFEPGALPTAEAVRFSLGVRVAKEDGNSGFDLGLQ-GNLLSLRRNVSVFIYC : 907
MLA18_1 : LVIRADGFHCMVDFHLDCGSATQILFEPGALPTAEAVRFSLGVRVAKEDGNGGFDLGLQ-GNLLSLRRNVSVFIYC : 907
MLA25_1 : LVIRADGFRCMVEFRLDCGSATQILFEPGALPRAESVVISLGVRVAKEDGNRGFDLGLQ-GNLLSLRRFVSVLIYC : 909
MLA38_1 : ---------- : -
RGH1bcd : ---------- : -
HcMLA1 : L----DGFRCLLRFSLDCGSAAQIMFEPGALPRVEIVIFSLGVRVAKEDGNCRFDLGLQ-GNLLSLRQRVWVKIYC : 894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MLA1 : GGARVGEAKEAEAAVRRALEAHPSHPRIYIQMRPHIAKGAHDDDLCEDEEEN---------- : 958
MLA2 : GGARVGEAKEAEAAVRRALDAHPNHPQIAIFMHPPIAEGAQDDDLM---------- : 951
MLA3 : GGATVGEAKEAEAVVRRALKADPHVYYISIQMRPRIAKGAHDDDLCEEWTDF---------- : 958
MLA6 : GGARVGEAKEAKAALRRAQEAHPDHLRIYIDMRPCIAEGAHDDDLCEGEEEN---------- : 956
MLA7 : GGARVGEAKEAEAAVRRALDAHPSHPPIYIEMRPHIAKGAHDDDLCEERRRTDF-------- : 959
MLA8 : GGARVGEAKEAEAAVRRALEAHPNHPRIYIYKTPCIAEGAHDDDLCVDGVDDDDFSDDEEN- : 967
MLA9 : GGARVGEAKEAEAAVRRALDAHPDHPQIAIFMHPPIAEGAQDDDLM---------- : 951
MLA10 : GGARVGEAKEAEAAVRRALDAHPNHPQIAIFMHPPIAEGAQDDDLM---------- : 951
MLA12 : GGARVGEAKEAEAAVRRALEAHPRHPRIYIDMRPDIQEGAHDDDLCENEDEGEN-------- : 961
MLA13 : GGARVGEAKEAEAAVRRALDAHPSHPPIYFEMRPHIAKGAHDDDLCEERRRTDF-------- : 959
MLA18_2 : GGARVGEAKEAEAALRRAHEAHPSHPRIYTQMRPRIAEGAHDDDLCEDEEEN---------- : 958
MLA19_1 : GGARVGEAKEAEAAVRRALEAHPRHPRIYIDMRPDIQEGAHDDDLCENEDEGEN-------- : 961
MLA22 : GGVRVWEAKEAEAAVRRALDAHPNHPQIIISMRTRIAEGAHDDDLCEDYDEVEN-------- : 959
MLA23 : GGATVGEAKEAEAVVRRALDAHPNHPQIAIFMHPPIAEGAQDDDLM---------- : 952
MLA27_1 : GGARVGEAKEAEAAVRRALEAHPNHPRIYIDMMPRIAQGAHDDDLCEDKVEN---------- : 957
MLA27_2 : GGARVGEAKEAEAAVRRALEAHPNHPRIYIDMMPRIAQGAHDDDLCEDEVDDDGDFFW---- : 963
MLA28 : GGVRVGEAKETEAAVRRALEAHPNHRRFYIQMRPHIAEGAHDDDLCVDEVDDDDFSDDEEN- : 967
MLA30_1 : GGARVGEAKEAEAAVRRALDAHPSHPPIYIEMRPHIAKGAHDDDLCEERRRTDF-------- : 959
MLA31_1 : GGARVGEAKEAEAAVRRALEAHPRHPRIYIDMRPDIQEGAHDDDLCENKDEGEN-------- : 954
MLA32 : GGARVGEAKEAEAAVRRAHEA------ACIQMRPRIAEGAHDDDLCEDEVDDDGDFFW---- : 957
MLA34 : GGARVGEAKEAEAAVRHALDAHSNHPAIAISMFPYIAEGAQDDDLM---------- : 952
MLA35_1 : GGARVGEAKEAEAAVRRALDAHPNHPQIAIFMHPPIAEGAQDDDLM---------- : 952
MLA36_1 : GGARVGEAKEAEAAVRRALDAHPSHPPIYIEMRPHIAKGAHDDDLCEERRRTDF-------- : 959
MLA37_1 : GGARVGEAKEAEAAVRRALDAHPDHPQIAIFMDPPIAEGAQDDDLM---------- : 950
MLA39_1 : RGARVGEAKEAEAALRRALEAHPSHPHIYIQMRPHIAEGAHDDDLCEEWTDF---------- : 958
MLA16_1 : RGARVGEAKEAEAAVRRALEAHPSHPRIYIQMRPRIAEGAHDDDLCEEWTDF---------- : 959
MLA18_1 : RGARVGEAKEAEAAVRRALEAHPSHPRIYIQMRPRIAEGAHDDDLCEEWTDF---------- : 959
MLA25_1 : GGARVGEAKEAEAAVRRALEAHPSHPRIYIDMRPDIQEDAHDDDLCENEDEVEN-------- : 963
MLA38_1 : ---------- : -
RGH1bcd : ---------- : -
HcMLA1 : GGATVGEAKEAEAAVRLALDSHPNHPPIYIEMRPRIQEDAHDDDLCKDMTEN---------- : 946

6.2. Estimated parameters of three different groups of MLA cDNAs

Log-likelihood values and parameter estimates under models of variable ω ratios for different sets of MLA cDNAs.

Model	**l** [a]	**Estimates of parameters** [b]
28 MLA cDNAs and their NJ tree		
M0	-12561.96	ω=0.627
M1	-12132.73	P_0=0.785, P_1=0.215, ω_0=0.0804, ω_1=1.000
M2	-11835.96	P_0=0.718, P_1=0.226, P_2=0.0560, ω_0=0.111, ω_1=1.000, ω_2=7.525
M3	-11805.85	P_0=0.870, P_1=0.110, P_2=0.019, w_0=0.209, ω_1=3.148, ω_2=14.628
M7	-12168.24	P=0.123, q=0.268
M8	-11841.93	P_0= 0.940, p=0.230, q=0.472, P_1= 0.060, ω=7.411
25 MLA cDNAs and their NJ tree		
M0	-10796.77	ω=0.612
M1	-10356.85	P_0=0.801, P_1=0.199, ω_0=0.042,ω_1=1.000
M2	-10057.65	P_0=0.731, P_1=0.217, P_2=0.052, ω_0=0.051, ω_1=1.000, ω_2=9.128
M3	-10028.68	P_0=0.872, P_1=0.106, P_2=0.022, $\omega_0$0=0.138, ω_1=3.337, ω_2=16.399
M7	-10370.93	P=0.015, q=0.049
M8	-10060.79	P_0=0.947, p=0.062, q=0.158, P_1=0.053, ω=9.256
19 MLA cDNAs and their NJ tree		
M0	-9353.71	ω=0.618
M1	-9057.47	P_0=0.756, P_1=0.244, ω_0=0.031, ω_1=1.000
M2	-8830.03	P_0=0.659, P_1=0.297, P_2=0.043, ω_0=0.011, ω_1=1.000, ω_2=10.423
M3	-8818.66	P_0=0.846, P_1=0.128, P_2=0.026, ω_0=0.126, ω_1=2.652, ω_2=15.242
M7	-9074.23	P=0.005, q=0.008
M8	-8830.14	P_0=0.956, p=0.013, q=0.027, P_1=0.044, ω=10.330

25 MLA cDNAs and their MP tree

M0	-10758.67	ω=0.592
M1	-10334.21	P_0=0.798, P_1=0.202, ω_0=0.043, ω_1=1.000
M2	-10052.92	P_0=0.736, P_1=0.209, P_2=0.055, ω_0=0.0541, ω_1=1.000, ω_2=8.645
M3	-10032.65	P_0=0.870 P_1=0.106, P_2=0.027, ω_0=0.135, ω_1=3.134, ω_2=14.820

M7	-10348.69	P=0.014, q=0.027
M8	-10055.75	P_0=0.944, p=0.074, q= 0.200, P_1=0.056, ω = 8.670

LRR domain of 25 MLA cDNAs and their NJ tree (starting at position 2100)

M0	-4654.61	ω=0.855
M1	-4462.91	P_0=0.687, P_1=0.313, ω_0=0.067 ω_1=1.000
M2	-4357.69	P_0=0.629, P_1=0.256, P_2=0.115 ω_0=0.090, ω_1=1.000, ω_2=4.977
M3	-4345-21	P_0=0.725, P_1=0.208, P_2=0.067, ω_0=0.158, ω_1=2.212, ω_2=7.671
M7	-4478.85	P=0.121, q=0.194
M8	-4362.11	P_0=0.847, P=0.230, q=0.471, P_1=0.153, ω=4.666

[a]Log-likelihood values. [b]Nonsynonymous-synonymous substitution ratio ($\omega = K_a/K_s$) averaged over sites.

6.3. Detailed protocol of the transient particle bombardment assay

Preparations:
Put plants into growth chamber six days before
1% Phytagar/Benzimidazole plates (Sarstedt 100 mm square plates)
Wash gold particles: Weight gold particles (0.003g/shot) in a 1.5ml Eppendorf tube. Resuspend gold particles in 70% EtOH, vortex for 5 min, sediment particles for 15 min on the bench. Wash particles 3 times with 1 ml MilliQ water (spin down, discard supernatant, resuspend in 50 + 950 µl MilliQ water, vortex for 1 min, sediment 1 min, spin down etc.).Resuspend particles in 50 µl 50% glycerol/3 mg gold (e.g. 4 shots = 12 mg resuspended in 200 µl glycerol), vortex for 5 min, store at -20°C until use.

Bombardment day
Let the agar plates incubate at RT (don't put leaves on cold plates!), 100% EtOH ("DNA quality"), $CaCl_2$ (stored at -20°C), store on ice, spermidine (stored in -20°C room): add to15.7 µl aliquot 984 µl MilliQ water to achieve a 0.1M solution) store on ice, take out rupture discs and place them on a tissue paper; 1 disc of 900 psi or 2 discs of 450 psi per shot plus 1 test shot)

Coating of the gold particles
(1) Make DNA premix (1.5 µg DNA/construct; max. 5 µg DNA in total)
(2) Dilute DNA with MilliQ water in a total volume of 20 µl.
(3) Take gold particles from -20°C, vortex for at least 1 min, sonicate for 1 min, mix by pipetting up and down, aliquot into 1.5ml Eppendorf tubes (vortex prior to each pipetting; use 50 µl / shot). Add 100 µl MilliQ water to each gold aliquot.
(4) During vortexing, add 30 µl Spermidine 0.1 M to each sample, sonicate for 10 sec.
(5) Add DNA premix to each sample, mix by pipetting up and down.
(6) During CONSTANT vortexing, add dropwise 75 µl $CaCl_2$ to each sample. Incubate at RT for 10 min. Keep on ice afterwards.
(7) Spin down particles, discard supernatant (as much as possible; particles stick now quite well), resuspend in 50 + 950 µl EtOH 100%, vortex for 1 min, sediment for 1 min.
(8) Repeat step (1) (take care not to discard too much gold). After vortexing, don't sediment particles, but store tubes horizontally on ice until step (3).
(9) Cutting the leaves on the plates. Place metal bars on the upper and lower end of the leaves.
(10) Centrifuge particles, discard supernatant, resuspend in 35 µl EtOH 100%, store on ice.

Bombardment
(1) Open Helium bottle, open working pressure valve to 1300 psi, switch on bombardment machine and vacuum pump.
(2) Test shot: Dip rupture disc into isopropanol, place it still wet in the rupture disc holder (use the pointed tweezers), remove air bubbles if there are any. Screw hepta adaptor into the machine, fix it with a wrench. Close the door and make sure that the security locking is really dip rupture disc into isopropanol, place it still wet in the rupture disc holder (use the pointed tweezer tweezers), remove air bubbles if there are any. Set the vacuum to 27 mmHg (put middle switch from mid position up to VAC position. When the pressure reaches 27 mmHg, move the switch to the HOLD position. Press and keep the FIRE switch pressed and watch the pressure gauge; check if the disc bursts at the correct pressure (900 psi). Release the switch immediately after the burst and put the vacuum switch back to the mid position to release the vacuum.
(3) Place 7 Macrocarrier discs into the holder and fix them with the red lid.

(4) Vortex gold particles well, distribute them on the Macrocarries only "inner circle" by pipetting 4 µl of the suspension to each disc (mix again and again by vortexing and pipetting up and down). Distribute remaining particles. If they stick to the tube wall, scrape them off with the pipette tip.
(5) Put the rupture disc (900 psi) into its holder and fix it in the machine (step 2).
(6) When the Macrocarriers are dry, place the stopping screen on the stopping screen holder and add carefully on top the Macrocarrier holder (turn it upside down).
(7) In the 2° shelf from top, place the stopping screen and Macrocarrier holder set.
(8) Put the plate with the leaves on the target shelf, add 2 metal bars on the side to fix the two metal bars that were already put, insert the target shelf into the 2° shelf from bottom.
(9) Close the door and bombard as described in step 2. Remove the 2 additional metal bars and soak up water droplets on leaves if there are any.

After bombardment:
Close the He bottle (clockwise). Apply a vacuum of ca. -15 mmHg to the empty chamber.
Press the FIRE button until the pressure in the bottle and the working pressure are no more going down. Open the working pressure valve (turn 3 times). Release the vacuum (mid position), switch off bombardment machine and vacuum pump. Clean the bombardment machine and hepta-adapter with 70% EtOH.

Infection with powdery mildew
Put the plates in a transparent box (lid of the plates and the box slightly open) and incubate them for 4h at 20°C, 80% humidity, medium light intensity. Inoculate the leaves with the corresponding isolate, remove the metal bars and put the plates back to the incubator for 44 h.

GUS staining
The GUS staining is performed 48h after bombardment.
(1) Defreeze GUS staining solution and warm it at 20-37°C (not warmer!).
(2) Place the metal bars back to the leaves, cut the leaves in the middle and cut away the ends that are below the bars (are anyway not bombarded). The cutting works best with a Japanese knife. Put the leaves of 1 shot in a tube and cover them with the GUS staining solution.
(3) Vacuum infiltrate the leaves until there is no air left (leaves turn dark green).
(4) Incubate o/n at 37°C.
(5) Discard GUS solution (non halogenic waste). Add destaining solution.
(6) Before counting, put the leaves in water. For long-term storage: in destaining solution

Destaining solution
Stock solution: 50% glycerol, 25% lactic acid, 25% water
Working solution: dissolve 1 volume stock solution in 2 volumues 100% Ethanol.

2.5 M $CaCl_2$
$CaCl_2 \cdot 2\ H_2O$, Mr = 147.02 g/mol, Fluka 21097 (internal code: C4)
20 ml: Dissolve 7.351g $CaCl_2$ in MilliQ water, adjust volume to 20 ml, filter sterilize, aliquot (e.g. 0.5 ml) in eppendorf tubes and store at -20°C.

Spermidine (6.368M)
Spermidine; Sigma S2626, 1 g, store at 4°C (not on stock; order freshly shortly before use)
Melt Spermidine stock at 65°C, put 15.7 µl aliquots in sterile eppendorf tubes (at sterile conditions). Store at -20°C. Long exposure to Spermidine can cause slight allergic reactions (better use the vertical laminar flow).

Benzimidazole 500 ppm
Benzimidazole; Fluka 12250 (internal code: B3),
1 litre: Dissolve 0.5g Benzimidazole in 1 litre autoclaved ddH_2O.

Phytagar plates
Plates: Sarstedt Petri Dish 100 mm SQ PS, No. 82.9923.422
0.75% Phytagar (Agar-Agar, Erne Chemie 80224, internal code: A21)
30 ppm Benzimidazole
Pour 50ml of this medium per plate and store them at 4°C.

0.5M HNa_2O_4P
$HNa_2O_4P \cdot 12\ H_2O$, Mr = 358.14 g/mol, Merck 1.06579.1000 (internal code: N9) (Alternatively, you can use HNa_2O_4P water free (Mr = 141.96 g/mol, N17))
250 ml: Dissolve 44.768g $HNa_2O_4P \cdot 12\ H_2O$ in ddH_2O (faster at 37°C), adjust volume to 250 ml. Autoclave.

0.5 M NaH_2PO_4
$NaH_2PO_4 \cdot H_2O$, Mr = 137.99 g/mol, Merck 1.06346.1000 (internal code: N6)
250 ml: Dissolve 17.249g $NaH_2P0_4 \cdot H_2O$ in ddH_20, adjust volume to 250 ml. Autoclave.

Glycerol 50%
Glycerol anhydrous, Fluka 49770 (internal code: G2). Dilute Glycerol with MilliQ water in a 1:1 ratio, filter sterilize and store at -20°C in small (e.g. 1ml) aliquots.

GUS staining solution
Add all chemicals in the order given above. Add Triton x-100 while constantly stirring. Aliquot the solution into 50ml falcon tubes and store at -20°C in the dark (X-Gluc, $K_4(CN)_6Fe \cdot 3H_20$ and $K_3(CN)_6Fe$ are photosensitive).

	final conc.	**1.0 L**	
X-Gluc (*)	1.0 mg/ml	1.0 g	Biosynth AG B-7300
Methanol	20 % (v/v)	200 ml	
Na-EDTA pH 8.0	10 mM	20 ml of 0.5 M	
Na_xPO_4 pH 6.5 (**)	0.1 M	200 ml of 0.5 M	
ddH_20			
KaliumhexacyanoferrateII	5 mM	2.112 g	Fluka 60280
Kaliumhexacyanoferrate III	5 mM	1.646 g	Fluka 60300
Triton x-100	0.1 % (v/v)	1 ml	Fluka 93420

* 5-Bromo-4-chloroyl-3-indoxl-beta-D-glucuronic acid, cyclohexylammonium salt acid)
** How to prepare the Na_xP04 buffer: Add ca. 120 ml of Na_2HPO_4 0.5M (pH ~4.2) into a beaker, add NaH_2PO_4 0.5M until the pH reaches 6.5 (ca. 100 ml). Use from this mixture 200ml.

6.4. Yeast media used for the yeast-two hybrid assay

SD medium (stock plates)

0.68 g	Difco™ Yeast Nitrogen Base (BD, #233520)
2 g	$(NH_4)_2SO_4$
0.56 g	Drop-out Medium –UHWL (Sigma, Y2001-20G)
8 g	agar
400 ml	V_{tot}

After autoclaving, 40 ml 20% glucose (sterile filtrated, store at 4°C) were added.

SD medium (test plates)

0.68 g	Difco™ Yeast Nitrogen Base (BD, #233520)
2 g	$(NH_4)_2SO_4$
0.56 g	Drop-out Medium –UHWL (Sigma, Y2001-20G)
4 g	Raffinose
8 g	agar
400 ml	V_{tot}

After autoclaving, 40 ml 20% galactose (sterile filtrated, store at 4°C), 40 ml BU salts and 4 ml X-Gal (20 mg/ml) were added.

Stock solutions:

BU-salts:

34.1 g	$NaH_2PO_4 \times H_2O$
105.9 g	$Na_2HPO_4 \times H_2O$
1000 ml	V_{tot}

adjust to pH 7, autoclave, store at RT.

X-Gal (20 mg/ml)

400 mg	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside solved in 20 ml N,N-dimethylformamide, stored in the dark, -20°C.

6.5. In vitro transcription to generate the three viral RNA products

T7 mMessage mMaschine Kit (Ambion, Catalog #1344) was used for *in vitro* transcription. For 20 plants, 20 µl of each of the three BSMV subgenomes will be produced:

4 µl	ddH_2O (RNAse free)
2 µl	linear template DNA (500 ng/µl)
2 µl	10x reaction buffer
10 µl	2xNTP/CAP
2 µl	Enzyme mix
20 µl	

FES buffer

1%	Betonite (Fluka, #11959)
	resuspended in water with sufficient boil and sonication cycles

To the Betonite solution were added:

20 ml	10x GP (0.5 M glycine and 0.3 M K_2HPO_4, sterile filtrated)
1%	sodium pyrophosphate
1%	Celite (Fluka, #22141)
100 ml	V_{tot}

The pH was adjusted to 8.5-9.0 with phosphoric acid and autoclaved for sterilization

6.6. List of primers used in chapter 2

MLA cDNAs	Forward Primer Reverse Primer	Sequences
2	sbi185	5'-GGGCGCGCCATACGGAGATCTCGTCCTCCTGCTCTC-3'
	sbi299	5'-GCTCGAGCGGCCGCTCACATTAAATCGTCATCTTGAGC-3'
3	sbi185	5'-GGGCGCGCCATACGGAGATCTCGTCCTCCTGCTCTC-3'
	spa011	5'-GGGCGGCCGCGATACAGTATGAATCCTTGTACG-3'
8	sbi184	5'-GGGCGCGCCAGACGGAGATCTCGTCCTCCTGCTCTC-3'
	sse017	5'-GCTCGAGCGGCCGCGATCAGTTCTCCTCATCATC-3'
9	sbi184	5'-GGGCGCGCCAGACGGAGATCTCGTCCTCCTGCTCTC-3'
	spa07	5'-GGGCGGCCGCGGTCAGAAATCGATACTCGAATCAG-3'
16-1	sse035	5'-GGGCGCGCCATGGAGGTCGTCACGGGTGC-3'
	sbi297	5'-GCTCGAGCGGCCGCTCAGAAATCAGTCCACTCCTC-3'
18-1	sse035	5'-GGGCGCGCCATGGAGGTCGTCACGGGTGC-3'
	sbi297	5'-GCTCGAGCGGCCGCTCAGAAATCAGTCCACTCCTC-3'
18-2	sbi185	5'-GGGCGCGCCATACGGAGATCTCGTCCTCCTGCTCTC-3'
	sbi295	5'-GCTCGAGCGGCCGCTCAGTTCTCCTCCTCGTCC-3'
19-1	sbi185	5'-GGGCGCGCCATACGGAGATCTCGTCCTCCTGCTCTC-3'
	sbi300	5'-GCTCGAGCGGCCGCTCAGTTCTCACCCTCGTCC-3'
22	sbi184	5'-GGGCGCGCCAGACGGAGATCTCGTCCTCCTGCTCTC-3'
	spa09	5'-GGGCGGCCGCGTAACCCCGCGCCCCTGCT-3’
23	sbi184	5'-GGGCGCGCCAGACGGAGATCTCGTCCTCCTGCTCTC-3'
	sbi299	5'-GCTCGAGCGGCCGCTCACATTAAATCGTCATCTTGAGC-3'
25-1	sse065	5'-GGGCGCGCCATGAATATTGTCACGGG-3'
	sbi301	5'-GCTCGAGCGGCCGCTCAGTTCTCGACCTCGTC
27-1	sbi185	5'-GGGCGCGCCATACGGAGATCTCGTCCTCCTGCTCTC-3'
	sse067	5'- GCTCGAGCGGCCGCTCATCACCAAAAAAAATCACC-3’
27-2	sbi185	5'-GGGCGCGCCATACGGAGATCTCGTCCTCCTGCTCTC-3'
	sse021	5'-GCTCGAGCGGCCGCCCTGTGTGTCCCTCCTACTG-3'
28	sbi185	5'-GGGCGCGCCATACGGAGATCTCGTCCTCCTGCTCTC-3'
	sse017	5'-GCTCGAGCGGCCGCGATCAGTTCTCCTCATCATC-3'
30-1	sbi185	5'-GGGCGCGCCATACGGAGATCTCGTCCTCCTGCTCTC-3'
	sbi298	5'-GCTCGAGCGGCCGCTCAGAAATCAGTTCTCCTCCTC-3'
31-1	sbi184	5'-GGGCGCGCCAGACGGAGATCTCGTCCTCCTGCTCTC-3'
	sse016	5'-GCTCGAGCGGCCGCTCAGTTCTCGCCCTCGTCC-3'
32	sbi185	5'-GGGCGCGCCATACGGAGATCTCGTCCTCCTGCTCTC-3'
	sse021	5'-GCTCGAGCGGCCGCCCTGTGTGTCCCTCCTACTG-3'
34	sbi185	5'-GGGCGCGCCATACGGAGATCTCGTCCTCCTGCTCTC-3'
	sbi299	5'-GCTCGAGCGGCCGCTCACATTAAATCGTCATCTTGAGC-3'
35-1	sbi184	5'-GGGCGCGCCAGACGGAGATCTCGTCCTCCTGCTCTC-3'
	sbi299	5'-GCTCGAGCGGCCGCTCACATTAAATCGTCATCTTGAGC-3'
36-1	sbi185	5'-GGGCGCGCCATACGGAGATCTCGTCCTCCTGCTCTC-3'
	sbi180	5'-CATGCATGGCGGCCGCATGCAATGTGAGTCGCTC-3'
37-1	sbi185	5'-GGGCGCGCCATACGGAGATCTCGTCCTCCTGCTCTC-3'
	sbi299	5'-GCTCGAGCGGCCGCTCACATTAAATCGTCATCTTGAGC-3'
38-1	sse064	5'-GGGCGCGCCAGCTGCTCACGGAGGTCGTC-3'
	sbi298	5'-GCTCGAGCGGCCGCTCAGAAATCAGTTCTCCTCCTC-3'
39-1	sbi184	5'-GGGCGCGCCAGACGGAGATCTCGTCCTCCTGCTCTC-3'
	sbi297	5'-GCTCGAGCGGCCGCTCAGAAATCAGTCCACTCCTC-3'

Other primers used

Name	Sequence
sbi178	5’-CCACGCAGATATTGTTTG-3’
sbi327	5’-CTTGGATCCTGATGCTAGGCAGTCGGCTAATCAC-3’
sbi398	5’-TGATGCGGCCGCAAATCCTTCGGCCACCCAC-3’
sbi480	5’-GGATCC$(T)_{31}$-3’
sse011	5’-GATGGATCCTGCCATTTAAAAGAAGCTCATCAC-3’
sse012	5’- GCGCAAGCTTCAACGGCTGCTAGTCATCC-3’
sse078	5’-CCTGTCGACCTTGTCCCGCGGCAC-3’

6.7. List of primers used in chapter 3

Name	Sequence
sse035	5’-GGGCGCGCCATGGAGGTCGTCACGGGTGC-3’
sse065	5’-GGGCGCGCCATGAATATTGTCACGGGGGC-3’
sse071	5’-GGCAGGATCCTAATGGAGGTCGTCACGGGTGC-3’
sse072	5’-GGAGCGGCCGCCTAGGCAGCGTGCATGCTCTCAA-3’
sse073	5’-GGCAGGATCCTAATGAATATTGTCACGGGGGC-3’
TJ60	5’-ATCAAGCTTGGATCCTGATGGATATTGTCACGGGT-3’
TJ61	5’-TCGCATGCGGCCGCTCACGCAGCGTTCATGGTGTT-3’
sse082	5’-GCATCCGCGGGAGCAGCTCGACAGCCAAGA-3’
sse083	5’-GCTGCCCTCATCAAGGTTGGTGAG-3’
sse084	5’-CCAACCTTGATGAGGGCAGC-3’
sse110	5’-AGGTTTGGCTTGATGATCTTC-3’
sse111	5’-GAAGATCATCAAGCCAAACCT-3’
sbi295	5’-GCTCGAGCGGCCGCTCAGTTCTCCTCCTCGTCC-3’
sse130	5’-GCATGCGGCCGCTCAGCTTCAAGCATAATCTGGAACATCGTATG GATAGCCACCGTTCTCCTCCTCGTCCTCAC-3’
sse143	5’-GTATGTCGTGTAGCTGATATGGTTCTGGACC-3’
sse144	5’-GGTCCAGAACCATATCAGCTACACGACATAC -3’
sse145	5’-CATGCTTGCCGTGTAGCTGATATGGTTC-3’
sse146	5’-GAACCATATCAGCTACACGGCAAGCATG-3’
sse147	5’-GCTTGCCGTGTAGCTGATATGGTTTTGG-3’
sse148	5’-CCAAAACCATATCAGCTACACGGCAAGC-3’
sse149	5’-GCTTGTCGTGTAGCTGATATGGTTCTGG-3’
sbi150	5’-CCAGAACCATATCAGCTACACGACAAGC-3’

6.8. List of all primers used in chapter 4

Name	Sequence
sse022	5’-CGGATAGCTAGCGGACTCCAGCCATTCAAAAC-3’
sse023	5’-CGGATACCGCGGTCATGCTGTTGTTGCTGCTGCCG-3’
sse024	5’-CGGATAGCTAGCGGTGAGCAAGGGCGAGGAG-3’
sse025	5’-CGGATACCGCGGTCACTTGTACAGCTCGTCC-3’
sse026	5’-GGATAGCTAGCGATAGGGTCAGTTGAAATTATTGAG-3’
sse027	5’-CGGATACCGCGGTCATAGGAACTGCTTTTTGTGGC-3’
sse028	5’-CCGCGGATGATCATGATGCTAGCATACTGTGAAGTGGTA AAAGAAATG-3
sse029	5’-GCTAGCATCATGATCATCCGCGGCGATAAGCGAACTATCGCTG-3’
sse030	5’-ATGGCCCACTACGTGAAC-3’
sse031	5’-TCGGAACTTTGGGAATCG-3’
sse032	5’-GCTAGCATCATGATCATCCGCGGTTCGAACGTGAACTCGCTTTGG-3’
sse033	5’-GCTAGCATCATGATCATCCGCGGTGACCTGCTGTTGAAGCGG-3’
sse042	5’-TTAAGGTACCACAACACACACAAGAGAAAGTAGCCATGGCCG CGGATGTATATGATGCTAGCATGCGAAGGTAAATACAGTAG-3’
sse043	5’-GCTAGCATCATGATCATCCGCGGTAAAAAAAAAAAAAAA ATGTTTGATCAGATCATTCAAATC-3’
sse044	5’-CCGCGGATGATCATGATGCTAGCTTAGAAACGGAAGAAGAATC-3’
sse045	5’-CCGCGGATGATCATGATGCTAGCATGCGAAGGTAAATA CAGTAG-3’
sbi216	5’-GAAAGTGTGCTGGCCTTTC-3’
sbi245	5’-GTTGCTGTTTAATTGTGAAGGG-3’
unil	5’-CGTTGTAAAACGACGGCCAGT-3’
revl	5’-CAGGAAACAGCTATGACCATG-3’
sse091	5’-GTTGGATCCTATCTCGTCCTCCAGCTCTC-3’
sse097	5’-ATGGCAGGGCGCGCCATGGTGAGCAAGGGC-3’
sse098	5’-GATAGCGGCCGCTCACTTGTACAGCTCGTCCAT-3’
sse099	5’-CACCATGGCGCGCCCTGCCATTAAATCGTCATCTTG-3’
sse100	5’-GCATGCGGCCGCTCAGCTTCAAGCATAATCTGGAACATCGTATG GATAGCCACCCATTAAATCGTCATCTTGAG-3’
sse102	5’-GTCACCATGGATGGACTCCAGCCATTCAAA-3’
sse103	5’-GCATGCGGCCGCTCACAAATCTTCTTCAGAAATCAACTTT TGTTCTGCTGTTGTTGCTGCTGCCGG-3’
sse104	5’-GTCACCATGGATGGAACAAAAGTTGATTTCTGAAGAAGA TTTGGACTCCAGCCATTCAAAACC-3’
sse105	5’-GCATGCGGCCGCTCATGCTGTTGTTGCTGCTG-3’
sse106	5’-GTCACCATGGATGATAGGGTCAGTTGAAAT-3’
sse107	5’-GCATGCGGCCGCTCACAAATCTTCTTCAGAAATCAACTT TTGTTCTAGGAACTGCTTTTTGTGGC-3’
sse108	5’-GTCACCATGGATGGAACAAAAGTTGATTTCTGAAGAAGAT TTGATAGGGTCAGTTGAAATTATTG-3’
sse109	5’-GCATGCGGCCGCTCATAGGAACTGCTTTTTGTGGC-3’
sse110	5’-AGGTTTGGCTTGATGATCTTC-3’
sbi184	5'-GGGCGCGCCAGACGGAGATCTCGTCCTCCTGCTCTC-3'
sbi299	5’- GCTCGAGCGGCCGCTCACATTAAATCGTCATCTTGAGC-3’

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Contributions by a Master student

Data generated by Simon Schwizer in his master thesis "Functional analysis of *Mla18-1* and *Mla25-1* and establishment of a heterologous *Mla/AVR$_A$* expression system" were included in this thesis. In chapter 3: He generated the domain swap constructs Swap2, Swap3, Swap5 and Swap6 (Chapter 3.4.1.), performed the transient overexpression experiments shown in Figure 10 and did the immunoblotting experiments mentioned under 3.4.8. The expression of the autoactive mutants in agroinfiltrated *N. benthamiana* shown in Figure 12 were also part of his master thesis. In chapter 4: He generated the myc-tagged effector constructs (Chapter 4.3.3.1.), performed all Gateway cloning experiments of pENTR plasmids into expression vectors (Chapter 4.3.3.2.), isolated and sequenced *Mla9* from cDNA (Chapter 4.3.4.) and did all expression experiments in *N. benthamiana* by agrobacterial infiltration (Figures 15 and 16).

Abbreviations

A6	powdery mildew isolate A6
aa	amino acid
Amp	ampicillin
A. tumefacines	*Agrobacterium tumefaciens*
ATP	Adenosintriphosphate
Apaf-1	apoptotic protease activating factor 1
Avr	Avirulence
BAK1	BRI1-associated receptor kinase 1
Bri1	brassinosteroid insensitive 1
Bgh	*Blumeria graminis* f. sp. *hordei*
bp	base pairs
BSA	bovine serum albumin
BSMV	barley stripe mosaic virus
CC	coiled-coil
DNA	deoxyribonucleic acid
cDNA	complementary DNA
cv.	cultivar
DMSO	dimethylsulfoxide
dNTP	desoxynucleotidetriphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetate
et al.	et alii (and others)
EtOH	ethanol
f. sp.	forma specialis
g	gram
h	hours
HA	hemagglutinin epitope
HR	hypersensitive reaction; programmed cell death
HSP70	heat shock protein 70
GFP	green fluorescent protein
in vitro	in the test tube; it refers to the absence of whole host organism
in vivo	in living cells
IPTG	isopropyl β-D-thiogalactopyranoside
IT	infection type
K1	powdery mildew isolate K1
KAc	potassium acetate
Kan	kanamycin
l	litre
LB	Luria-Bertani
LRR	leucine rich repeat
M	molar (mol/l)
MAMP	microbe-associated molecular pattern
MAPK	mitogen-activated protein kinase
min	minutes
Mla	mildew resistance locus a
Mlk	mildew resistance locus k
Mlo	mildew resistance locus o
mRNA	messenger RNA

N. benthamiana	*Nicotiana benthamiana*
NBS	nucleotide binding site
OD_{600}	optical density at 600 nm
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDB	protein data bank
PDS	phytoene desaturase
PEG	polyethylene glycol
pv.	pathovar
ubi	ubiquitin
RAR1	required for *Mla12* resistance
RPP	resistance to *Peronospora parasitica*
RPS	resistance to *Pseudomonas syringae*
RT	room temperature
RT PCR	reverse transcription PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SGT1	suppressor of G-two allele of skp1
Skp1	suppressor of kinetochore protein 1
sp.	species
spp.	all species belonging to the genus
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)aminomethane
TIR	Toll-interleukin receptor
TLR3	Toll-like recptor 3
v/v	volume per volume
VIGS	virus-induced gene silencing
VIGE	virus-induced gene expression
WRKY	transcription factor family containing the conserved motif “WRKY”
wt	wiltd type
w/v	weight per volume
YFP	yellow fluorescence protein
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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